Volume 2

Effects of Polyphenol-Rich Foods on Human Health

Edited by

Giuseppe Grosso

Printed Edition of the Special Issue Published in *Nutrients*

www.mdpi.com/journal/nutrients

MDPI

Effects of Polyphenol-Rich Foods on Human Health

Volume 2

Effects of Polyphenol-Rich Foods on Human Health

Volume 2

Special Issue Editor

Giuseppe Grosso

MDPI • Basel • Beijing • Wuhan • Barcelona • Belgrade

Special Issue Editor
Giuseppe Grosso
Azienda Ospedaliera Universitaria Policlinico Vittorio Emanuele
Italy

Editorial Office
MDPI
St. Alban-Anlage 66
Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Nutrients* (ISSN 2072-6643) from 2017 to 2018 (available at: http://www.mdpi.com/journal/nutrients/special_issues/polyphenol_rich_foods)

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

LastName, A.A.; LastName, B.B.; LastName, C.C. Article Title. *Journal Name* **Year**, *Article Number*, Page Range.

Volume 2
ISBN 978-3-03897-141-2 (Pbk)
ISBN 978-3-03897-142-9 (PDF)

Volume 1–3
ISBN 978-3-03897-145-0 (Pbk)
ISBN 978-3-03897-146-7 (PDF)

Articles in this volume are Open Access and distributed under the Creative Commons Attribution (CC BY) license, which allows users to download, copy and build upon published articles even for commercial purposes, as long as the author and publisher are properly credited, which ensures maximum dissemination and a wider impact of our publications. The book taken as a whole is © 2018 MDPI, Basel, Switzerland, distributed under the terms and conditions of the Creative Commons license CC BY-NC-ND (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

About the Special Issue Editor ix

Preface to "Effects of Polyphenol-Rich Foods on Human Health" xi

Keiko Unno, Shigenori Noda, Yohei Kawasaki, Hiroshi Yamada, Akio Morita, Kazuaki Iguchi and Yoriyuki Nakamura
Reduced Stress and Improved Sleep Quality Caused by Green Tea Are Associated with a Reduced Caffeine Content
Reprinted from: *Nutrients* **2017**, *9*, 777, doi: 10.3390/nu9070777 1

Teresa Esposito, Carla Schettino, Paola Polverino, Salvatore Allocca, Laura Adelfi, Alessandra D'Amico, Guglielmo Capaldo, Bruno Varriale, Anna Di Salle, Gianfranco Peluso, Giuseppe Sorrentino, Giacomo Lus, Simone Sampaolo, Giuseppe Di Iorio and Mariarosa Anna Beatrice Melone
Synergistic Interplay between Curcumin and Polyphenol-Rich Foods in the Mediterranean Diet: Therapeutic Prospects for Neurofibromatosis 1 Patients
Reprinted from: *Nutrients* **2017**, *9*, 783, doi: 10.3390/nu9070783 14

Ivana R. Sequeira and Sally D. Poppitt
Unfolding Novel Mechanisms of Polyphenol Flavonoids for Better Glycaemic Control: Targeting Pancreatic Islet Amyloid Polypeptide (IAPP)
Reprinted from: *Nutrients* **2017**, *9*, 788, doi: 10.3390/nu9070788 31

Hwayoung Noh, Heinz Freisling, Nada Assi, Raul Zamora-Ros, David Achaintre, Aurélie Affret, Francesca Mancini, Marie-Christine Boutron-Ruault, Anna Flögel, Heiner Boeing, Tilman Kühn, Ruth Schübel, Antonia Trichopoulou, Androniki Naska, Maria Kritikou, Domenico Palli, Valeria Pala, Rosario Tumino, Fulvio Ricceri, Maria Santucci de Magistris, Amanda Cross, Nadia Slimani, Augustin Scalbert and Pietro Ferrari
Identification of Urinary Polyphenol Metabolite Patterns Associated with Polyphenol-Rich Food Intake in Adults from Four European Countries
Reprinted from: *Nutrients* **2017**, *9*, 796, doi: 10.3390/nu9080796 52

Qingyu Ma, Yan Guo, Liping Sun and Yongliang Zhuang
Anti-Diabetic Effects of Phenolic Extract from Rambutan Peels (*Nephelium lappaceum*) in High-Fat Diet and Streptozotocin-Induced Diabetic Mice
Reprinted from: *Nutrients* **2017**, *9*, 801, doi: 10.3390/nu9080801 66

Guo-Yi Tang, Xiao Meng, Ya Li, Cai-Ning Zhao, Qing Liu and Hua-Bin Li
Effects of Vegetables on Cardiovascular Diseases and Related Mechanisms
Reprinted from: *Nutrients* **2017**, *9*, 857, doi: 10.3390/nu9080857 78

Giuseppe Grosso, Agnieszka Micek, Justyna Godos, Andrzej Pajak, Salvatore Sciacca, Maira Bes-Rastrollo, Fabio Galvano and Miguel A. Martinez-Gonzalez
Long-Term Coffee Consumption Is Associated with Decreased Incidence of New-Onset Hypertension: A Dose–Response Meta-Analysis
Reprinted from: *Nutrients* **2017**, *9*, 890, doi: 10.3390/nu9080890 103

Haneen Amawi, Charles R. Ashby Jr., Temesgen Samuel, Ramalingam Peraman and Amit K. Tiwari
Polyphenolic Nutrients in Cancer Chemoprevention and Metastasis: Role of the Epithelial-to-Mesenchymal (EMT) Pathway
Reprinted from: *Nutrients* **2017**, *9*, 911, doi: 10.3390/nu9080911 . . . . **116**

Francesca Venturi, Chiara Sanmartin, Isabella Taglieri, Anita Nari, Gianpaolo Andrich, Erika Terzuoli, Sandra Donnini, Cristiano Nicolella and Angela Zinnai
Development of Phenol-Enriched Olive Oil with Phenolic Compounds Extracted from Wastewater Produced by Physical Refining
Reprinted from: *Nutrients* **2017**, *9*, 916, doi: 10.3390/nu9080916 . . . . **139**

Jace Schell, R. Hal Scofield, James R. Barrett, Biji T. Kurien, Nancy Betts, Timothy J. Lyons, Yan Daniel Zhao and Arpita Basu
Strawberries Improve Pain and Inflammation in Obese Adults with Radiographic Evidence of Knee Osteoarthritis
Reprinted from: *Nutrients* **2017**, *9*, 949, doi: 10.3390/nu9090949 . . . . **152**

Justyna Godos, Agnieszka Micek, Marina Marranzano, Federico Salomone, Daniele Del Rio and Sumantra Ray
Coffee Consumption and Risk of Biliary Tract Cancers and Liver Cancer: A Dose–Response Meta-Analysis of Prospective Cohort Studies
Reprinted from: *Nutrients* **2017**, *9*, 950, doi: 10.3390/nu9090950 . . . . **165**

Stan Kubow, Michèle M. Iskandar, Emiliano Melgar-Bermudez, Lekha Sleno, Kebba Sabally, Behnam Azadi, Emily How, Satya Prakash, Gabriela Burgos and Thomas zum Felde
Effects of Simulated Human Gastrointestinal Digestion of Two Purple-Fleshed Potato Cultivars on Anthocyanin Composition and Cytotoxicity in Colonic Cancer and Non-Tumorigenic Cells
Reprinted from: *Nutrients* **2017**, *9*, 953, doi: 10.3390/nu9090953 . . . . **182**

Chanmin Liu, Jieqiong Ma, Jianmei Sun, Chao Cheng, Zhaojun Feng, Hong Jiang and Wei Yang
Flavonoid-Rich Extract of *Paulownia fortunei* Flowers Attenuates Diet-Induced Hyperlipidemia, Hepatic Steatosis and Insulin Resistance in Obesity Mice by AMPK Pathway
Reprinted from: *Nutrients* **2017**, *9*, 959, doi: 10.3390/nu9090959 . . . . **199**

Francesca Danesi and Lynnette R. Ferguson
Could Pomegranate Juice Help in the Control of Inflammatory Diseases?
Reprinted from: *Nutrients* **2017**, *9*, 958, doi: 10.3390/nu9090958 . . . . **214**

Kumju Youn, Yoonjin Yu, Jinhyuk Lee, Woo-Sik Jeong, Chi-Tang Ho and Mira Jun
Polymethoxyflavones: Novel β-Secretase (BACE1) Inhibitors from Citrus Peels
Reprinted from: *Nutrients* **2017**, *9*, 973, doi: 10.3390/nu9090973 . . . . **237**

Michal Bijak, Angela Dziedzic, Ewelina Synowiec, Tomasz Sliwinski and Joanna Saluk-Bijak
Flavonolignans Inhibit IL1-β-Induced Cross-Talk between Blood Platelets and Leukocytes
Reprinted from: *Nutrients* **2017**, *9*, 1022, doi: 10.3390/nu9091022 . . . . **249**

Diego dos Santos Baião, Cyntia Silva de Freitas, Laidson Paes Gomes, Davi da Silva, Anna Carolina N. T. F. Correa, Patricia Ribeiro Pereira, Eduardo Mere Del Aguila and Vania Margaret Flosi Paschoalin
Polyphenols from Root, Tubercles and Grains Cropped in Brazil: Chemical and Nutritional Characterization and Their Effects on Human Health and Diseases
Reprinted from: *Nutrients* **2017**, *9*, 1044, doi: 10.3390/nu9091044 . . . . **260**

Nitin Shivappa, Justyna Godos, James R. Hébert, Michael D. Wirth, Gabriele Piuri, Attilio F. Speciani and Giuseppe Grosso
Dietary Inflammatory Index and Colorectal Cancer Risk—A Meta-Analysis
Reprinted from: *Nutrients* **2017**, *9*, 1043, doi: 10.3390/nu9091043 . . . . **289**

Yih Ho, Yu-Syuan Lin, Hsuan-Liang Liu, Ya-Jung Shih, Shin-Ying Lin, Ai Shih, Yu-Tang Chin, Yi-Ru Chen, Hung-Yun Lin and Paul J. Davis
Biological Mechanisms by Which Antiproliferative Actions of Resveratrol Are Minimized
Reprinted from: *Nutrients* **2017**, *9*, 1046, doi: 10.3390/nu9101046 . . . . **305**

Kok-Yong Chin and Kok-Lun Pang
Therapeutic Effects of Olive and Its Derivatives on Osteoarthritis: From Bench to Bedside
Reprinted from: *Nutrients* **2017**, *9*, 1060, doi: 10.3390/nu9101060 . . . . **316**

Justyna Godos, Dario Sinatra, Isabella Blanco, Serena Mulè, Melania La Verde and Marina Marranzano
Association between Dietary Phenolic Acids and Hypertension in a Mediterranean Cohort
Reprinted from: *Nutrients* **2017**, *9*, 1069, doi: 10.3390/nu9101069 . . . . **334**

About the Special Issue Editor

Giuseppe Grosso's research focuses on evidence-based nutrition, a recently emerged field as the bottom line of the Health Technology Assessment applied to food and nutrition. The main interests include the impact of dietary and lifestyle habits on common non-communicable diseases. In particular, he produced over 100 papers on the effects of dietary patterns (i.e., Mediterranean diet) and specific antioxidant-rich foods (i.e., coffee, tea), as well as individual antioxidants (i.e., polyphenols, n-3 PUFA) on cardiovascular and metabolic diseases, cancer, and depression. Dr. Grosso conducted his research on cohorts of individuals in both Mediterranean and non-Mediterranean countries collaborating with several research institutions. He is interested in evidence synthesis aimed to generate policy-oriented research in the area of public health nutrition. He is currently working as research fellow at Integrated Cancer Registry of Catania-Messina-Siracusa-Enna, southern Italy. He is a cum-laude graduated MD and PhD.

Preface to ”Effects of Polyphenol-Rich Foods on Human Health”

The global burden of non-communicable diseases (NCDs) has been rising over the last century, and among the main NCDs are cardiovascular diseases (CVDs), cancers and diabetes. Besides genetic, environmental, and social factors, exploring dietary factors influencing such conditions is of primary importance to better define effective strategies for reducing the burden of disease. In fact, higher adherence to healthy and equilibrated dietary patterns has been shown to be implicated in prevention of NCDs.

In recent years, polyphenols have received a great deal of attention due to their potential beneficial effects on human health. Contained in foods commonly consumed in all populations worldwide, polyphenols offer a range of beneficial effects and are contained not only in fruits and vegetables, characteristic components of healthy dietary patterns, but also in other plant-derived foods, such as tea, coffee, and cocoa, which only recently have been scientifically exploited as being beneficial for humans. In addition to the numerous biological properties, polyphenols have been indicated as being responsible for a decreased risk of several health outcomes. Numerous epidemiological studies have demonstrated the association between both polyphenols and polyphenol-rich foods intake and human health. For example, recent meta-analyses show that high consumption of polyphenols and polyphenol-rich foods decrease the risk of overall and CVD-related mortality, cancer, CVD, diabetes and neurodegenerative diseases. However, some studies reported null results, which could be at least partially explained by significant differences in dietary intake of polyphenols in different populations, differences in food processing (loss of phenolic content), absorption, bioavailability, and metabolism of polyphenols.

Several molecular mechanisms have been taken into account for the beneficial effects of polyphenols. The antioxidant effects of dietary polyphenols can be attributed to the regulation of redox enzymes through reducing reactive oxygen species (ROS) production and modulation of the II-phase enzymes responsible for the cellular oxidative response. Moreover, several studies have suggested that polyphenols may exert protective effects on cardio-metabolic health by reducing inflammatory response, lowering LDL oxidation and blood pressure, and by improving endothelial function. Finally, polyphenols may exert chemo-preventive effects through a variety of mechanisms, including elimination of carcinogenic agents, modulation of pathways responsible for cancer cell signaling and cell cycle progression, and by promotion of apoptosis.

Even though research is ongoing, further evidence is needed in order to better characterize dietary factors that may exert beneficial effects toward prevention of chronic diseases associated with oxidative stress and inflammation.

Giuseppe Grosso
Special Issue Editor

nutrients

MDPI

Article

Reduced Stress and Improved Sleep Quality Caused by Green Tea Are Associated with a Reduced Caffeine Content

Keiko Unno [1,2,*], Shigenori Noda [3], Yohei Kawasaki [3], Hiroshi Yamada [3], Akio Morita [4], Kazuaki Iguchi [1] and Yoriyuki Nakamura [2]

1. Department of Neurophysiology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan; iguchi@u-shizuoka-ken.ac.jp
2. Tea Science Center, Graduate Division of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka 422-8526, Japan; yori.naka222@u-shizuoka-ken.ac.jp
3. Division of Drug Evaluation & Informatics, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan; s15412@u-shizuoka-ken.ac.jp (S.N.); kawasaki.yohei.2r@kyoto-u.ac.jp (Y.K.); hyamada@u-shizuoka-ken.ac.jp (H.Y.)
4. Department of Functional Plant Physiology, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan; morita.akio@shizuoka.ac.jp

* Correspondence: unno@u-shizuoka-ken.ac.jp; Tel.: +81-54-264-5731

Received: 10 June 2017; Accepted: 16 July 2017; Published: 19 July 2017

Abstract: Caffeine, one of the main components in green tea, can interfere with sleep and block the effect of theanine. Since theanine, the main amino acid in tea leaves, has significant anti-stress effects in animals and humans, we examined the effects of green tea with lowered caffeine content, i.e., low-caffeine green tea (LCGT), on stress and quality of sleep of middle–aged individuals (n = 20, mean age 51.3 ± 6.7 years) in a double-blind crossover design. Standard green tea (SGT) was used as the control. These teas (≥300 mL/day), which were eluted with room temperature water, were consumed over a period of seven days after a single washout term. The level of salivary α-amylase activity (sAA), a stress marker, was significantly lower in participants that consumed LCGT (64.7 U/mL) than in those that consumed SGT (73.9 U/mL). Sleep quality was higher in participants that consumed a larger quantity of LCGT. In addition, a self-diagnostic check for accumulated fatigue was significantly lower in those participants that consumed LCGT than SGT. These results indicate that LCGT intake can reduce stress in middle-aged individuals and improve their quality of sleep. The reduction in caffeine is suggested to be a valid reason for enhancing the anti-stress effect of green tea.

Keywords: anti-stress effect; caffeine; green tea; middle-aged individuals; salivary α-amylase; sleep

1. Introduction

Green tea (*Camellia sinensis* (L.) Kuntze) is the most popular drink in Japan and Asian countries. Epidemiological and animal studies have demonstrated that the ingestion of green tea enhances a healthy life [1,2]. Green tea is mainly composed of catechins (8–20%), caffeine (2–4%), and amino acids (1–8%) [3]. Epigallocatechin gallate (EGCG) is the most abundant catechin, followed by epigallocatechin (EGC). Theanine (L-theanine, *N*-ethyl-L-glutamine) is the most common amino acid, but other amino acids such as arginine (Arg) and glutamic acid (Glu) are also found in tea leaves. Some mechanisms of action of theanine, which has significant anti-stress effects in animals and humans [4–7], have been proposed, as mentioned next. Theanine, when incorporated into the brain, reportedly acts on the glutamine (Gln) transporter, and inhibits the incorporation of extracellular Gln into neurons [8]. Gln converts to Glu with the assistance of glutaminase, and Glu can be decarboxylated into γ-amino butyric acid (GABA) in neurons. In the hippocampus of mice that

ingested theanine, the level of Glu was significantly reduced while the level of GABA increased [9], indicating that theanine modulates GABA production from Glu. In the brain, Glu is the main excitatory neurotransmitter while GABA is the main inhibitory neurotransmitter. Whereas suitable synaptic excitation is important, excessive excitation damages nerve cells and triggers neurodegenerative diseases [10], suggesting that the balance between Glu and GABA is important. In addition, we recently found that caffeine and EGCG suppressed the anti-stress effect of theanine while EGC and Arg retained these effects [11]. The balance between theanine, caffeine, catechins, and arginine (Arg) is suggested to be crucial for green tea to express its anti-stress effect [11]. Intervention with green tea is thought to help prevent the accumulation of stress, and to be a potential therapeutic strategy for a healthy life. However, the anti-stress effect of green tea remains unknown because it has not yet been clarified how components of green tea other than theanine influence the anti-stress effect of theanine.

Therefore, we prepared green tea with a lowered level of caffeine, low-caffeine green tea (LCGT), in which caffeine content was reduced to one-quarter to one-fifth of the level of standard green tea (SGT). Furthermore, we tried to suppress the elution of EGCG and caffeine. Since green tea is generally eluted with hot water, the eluate is rich in EGCG and caffeine. However, the solubility of EGCG and caffeine is low in room temperature water (EGCG is <5 mg/mL and caffeine is 22 mg/mL) [12]. On the other hand, the solubility of theanine is high (370 mg/mL), even at room temperature. When green tea is eluted with low temperature water, the relative ratio of theanine to EGCG and caffeine in eluate is higher than when eluted with hot water [13,14]. Therefore, in this experiment, we examined the anti-stress effect of SGT and LCGT that had been eluted with room temperature water. Indeed, the ingestion of LCGT that was steeped in room temperature water significantly suppressed the stress response in mice and in young individuals [11,12].

In this study, we examined the effect of green tea on stress responses and sleep parameters in middle-aged individuals who work in Japan. Since they drink ~1000 mL of green tea daily, drinking green tea was considered to be a basal condition. However, since there were individual differences in the amount and concentration of green tea, we requested participants to drink water for one week to washout caffeine before they consumed SGT or LCGT. In a crossover design, the effect of ingesting LCGT versus SGT was compared.

Salivary α-amylase activity (sAA), which is an oral cavity enzyme, was measured as a stress marker [15]. In humans and animals, two main systems in the body are involved in the stress response, the autonomic nervous system (ANS) and the hypothalamus-pituitary-adrenal axis. Measurement of sAA is a useful tool for monitoring ANS reactivity to stress [15]. This enzyme rapidly increases in response to physiological and psychosocial stress [16–18]. In addition, caffeine and a psychological stressor (20 min of mental arithmetic) are reported to increase sAA [19]. Since sAA activity is affected by sleeping time [20,21], the effects of SGT or LCGT intake on sleep parameters and the correlation between sAA levels and sleep parameters were examined. Sleep parameters were measured using a single-channel electroencephalogram (EEG). Furthermore, the effect of ingesting SGT and LCGT on subjective stress and fatigue were compared. To prevent health problems caused by overwork, the degree of fatigue accumulation in workers needs to be assessed. If ingestion of green tea can reduce chronic fatigue, then it may be considered a very useful health management tool. In this study, our objective was to examine the stress-reducing effect of green tea and to evaluate the effect of reducing the caffeine content in green tea on sAA, sleep, and subjective fatigue.

2. Materials and Methods

2.1. Preparation of LCGT

Tea leaves were collected in Kakegawa, Shizuoka, Japan. Fresh tea leaves were treated with a hot water shower at 95 °C for 180 s [11]. As a result of this treatment, caffeine was washed away from the tea leaves and at the same time the activities of the enzymes in the tea leaves were stopped. After

the hot water was removed from the tea leaves by centrifugation, the tea leaves were dried through a standard manufacturing process, namely rolling and drying.

One tea bag of SGT or LCGT (3 g of tea in each bag) was steeped in 500 mL of room temperature water in a water bottle. Tap water was used in this experiment. The participants prepared SGT or LCGT every morning and ingested it until the evening. Tea bags were left in water until all tea had been fully consumed. Similarly, after each day's work, participants drank both teas.

To measure tea components in the eluate, tea leaves of SGT or LCGT (3 g) were steeped in 500 mL of room temperature water for 0.5, 1, 3, and 6 h with occasional stirring.

2.2. Measurement of Tea Components by HPLC

SGT and LCGT eluates were measured by HPLC as described previously [11]. In brief, catechins and caffeine in the eluates were measured by HPLC (SCL-10Avp, Shimadzu, Kyoto, Japan; Develosil packed column ODS-HG-5, 150 × 4.6 mm, Nomura Chemical Co. Ltd., Seto, Aichi, Japan) according to the method of Horie et al. [22]. Catechins and caffeine were measured at 280 nm. Free amino acids in tea leaves were measured by HPLC as described above using homoserine as the internal standard [23]. Amino acids were detected at an excitation wavelength of 340 nm and at an emission wavelength of 450 nm using an RF-535 UV detector (Shimadzu, Japan). The relative standard deviation (RSD%) of precision and repeatability were <5.0%. The recoveries of catechins, caffeine, and free amino acids were 99 ± 4%, 98 ± 4%, and 98 ± 3%, respectively.

2.3. Participants

Twenty middle-aged individuals (n = 20, 11 male and 9 female, mean age and SD, 51.3 ± 6.7 years), who work at Kakegawa City Hall in Japan, received verbal and written information about the study and signed an informed consent form before entering the study. None of the participants indicated acute or chronic diseases, regular intake of medication, or habitual smoking. Although they drank green tea daily, they did not show symptoms of subjective insomnia due to the intake of green tea. Participants were instructed to drink mainly the test tea, and not to take theanine- and caffeine-rich beverages such as green tea, coffee, and black tea throughout the experiment. The study was conducted in accordance with the Declaration of Helsinki and Ethical Guidelines for Medical and Health Research Involving Human Subjects (Public Notice of the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare, 2015). The study protocol was approved by the Ethics Committee of the University of Shizuoka (No. 27-22). This study was registered at the University Hospital Medical Information Network (UMIN) (registration ID No. UMIN19411). The study period was from November to December 2015.

2.4. Procedure

This study was a double-blind crossover design (Table 1). Since the required sample size was calculated to be 18 participants per group, the sample size was set to 20 participants per group considering dropouts, totaling 40 participants. The participants drank water for one week to washout caffeine before they consumed LCGT or SGT. SGT was used as the control beverage. The grouping of which participants should drinking first was achieved by complete randomization. These teas (≥300 mL/day) were consumed over a period of seven days after a single washout. The primary outcome of this study was to observe the change in sAA. Sleeping hours and sAA of each participant were recorded in a questionnaire. Subjective stress at the same time every evening (~9 PM) was evaluated using visual analog scales (VAS: 0–10) from very relaxed to highly stressed and recorded in a questionnaire. The physical condition of participants was assigned an ordinal scale (5, very good; 4, good; 3, normal; 2, slightly poor; 1, poor). A self-diagnostic check for accumulated fatigue and work severity that was created by the Japanese Ministry of Health, Labor and Welfare, was carried out every Monday morning (~9 AM) and every Friday evening (~9 PM) throughout the test period. An overnight EEG was monitored for three days (from Tuesday to Thursday) in the test period.

Table 1. Experimental protocol.

Term (1 Term with 7 Days)	1	2	3	4
Green tea intake	Washout (water)	SGT or LCGT	Washout (water)	LCGT or SGT
	(Saturday~Friday)	(Saturday~Friday)	(Saturday~Friday)	(Saturday~Friday)
Measurement of α-amylase activity	(-)	2 times/day (morning and evening)	(-)	2 times/day (morning and evening)
Subjective stress and Physical condition	(-)	every evening	(-)	every evening
Sleep	(-)	3 nights/week (Tuesday~Thursday)	(-)	3 nights/week (Tuesday~Thursday)
Subjective fatigue	(-)	2 times/week (Mon morning and Fri evening)	(-)	2 times/week (Monday morning and Friday evening)

This study was conducted in a double-blind crossover design. Participants drank SGT or LCGT that was eluted with room temperature water for a period of seven days after a single washout with water.

2.5. Measurement of sAA

To assess the physiological stress response, sAA was measured using a testing strip, and a colorimetric system (Nipro Co., Osaka, Japan) [24]. Briefly, the testing strip consisted of a collecting strip used to collect saliva and a reagent strip used to measure sAA. A substrate in the reagent strip, 2-chloro-4-nitrophenyl-4-*O*-β-D-galactopyranosylmaltoside is hydrolyzed by sAA in the presence of maltose, a competitive inhibitor. This reaction turns the color of the reagent strip from white to yellow, and the change is quantified using a colorimetric system (sAA monitor). One unit of activity (U) per mass of enzyme is defined as the production of 1 μmol of the reducing sugar, maltose, in 1 min (NC-IUBMB, 1992).

Saliva was measured twice a day on working days: the first time was in the morning within 0.5 h after waking up (sAA before work), and the second time was in the evening before dinner (sAA after work). Prior to sampling, participants washed their mouths with water. After saliva was collected for 30 s using a testing strip, the participants measured their own sAA. The mean values among each participant and the mean values between each green tea group were compared.

2.6. Measurement of EEG

EEG monitoring was achieved by using a single-channel EEG (Sleep Scope, SleepWell Co., Osaka, Japan) [25], as previously described for sleep scoring programs [26,27]. The channel located at approximately Fpz-M1 was recorded for the 1ch EEG system. Before sleep, participants attached two electrodes of the Sleep Scope EEG to their forehead and mastoid to collect electrophysiological signals. Measurements started when participants entered their beds. After participants woke up and exited their beds, measurements were stopped.

The data was analyzed at the SleepWell company (SleepWell Co., Osaka, Japan) and categorized into rapid eye movement (REM) sleep and non-REM sleep, which was again classified into light sleep (N1) or slow-wave sleep (N2+N3). The onset of sleep (SL) was defined as 5 min of continuous sleep. The onset of REM (REM SL) was the time from the onset of sleep to first REM sleep. Total sleep time (TST) was calculated as the total period of sleep (SPT) minus the time spent awake during the sleep period (WASO). Sleep efficiency (SE) was the ratio of TST to the time in bed (TIB). Early morning awakening (B2 WASO) was evaluated from the time spent awake during 2 h before final awakening. Average sleep cycle (AVE SLC) is the average time from the end of REM sleep to the end of the next REM sleep. The delta wave emerges mainly during non-REM deep sleep. This value is used as an indicator of sleep depth. First delta (1st δ) is the total delta power amount during non-REM sleep in the first sleep cycle.

2.7. Statistical Analysis

The results are expressed as the mean ± SEM. Differences in sAA were evaluated using one-way analysis of variance (ANOVA) followed by a Tukey–Kramer post hoc test for multiple comparisons. A paired *t*-test was carried out on the differences between sAA and several sleep parameters from SGT intake to LCGT intake. All statistical analyses were carried out using Statistical Analysis System (SAS 9.4, SAS Institute Inc., Cary, NC, USA). In each analysis, a p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Tea Components in SGT and LCGT

The content of caffeine in LCGT steeped in room temperature water was about 0.3~0.4 times that of SGT (Figure 1a). Although EGCG was the main catechin in tea leaves, EGC was the most abundant catechin in the eluate with room temperature water (Figure 1b). The total amount of catechins was almost the same between SGT and LCGT at all elution times, except 1 h. The amount of theanine in LCGT was about 1.2 times higher than in SGT (Figure 1c). Arg in LCGT was about 1.4 times higher than in SGT. The total amount of amino acids was 1.3 times higher in LCGT than in SGT after elution for 3 and 6 h. The concentration of each component increased with elution period, but the relative ratio was almost the same.

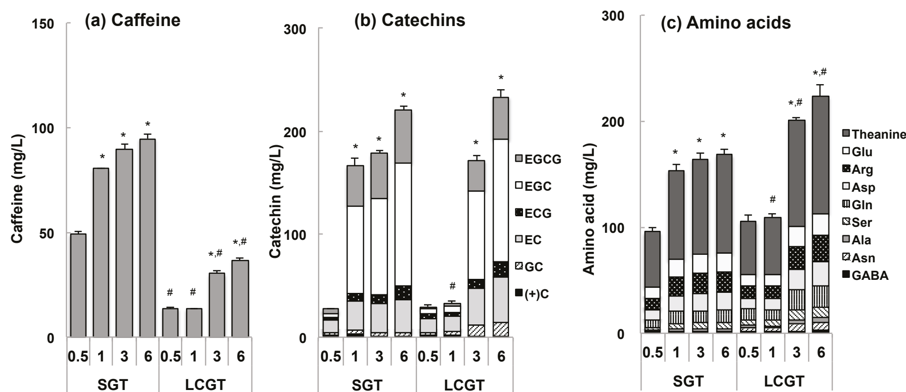


Figure 1. Tea components in SGT and LCGT. One tea bag of SGT or LCGT (3 g of tea in a bag) was steeped in 500 mL of room temperature water (0.5–6 h). Tap water was used in this experiment. Data are expressed as mean ± SD (n = 3). The data of SGT or LCGT was compared with that at 0.5 h, respectively (*, $p < 0.05$). In addition, LCGT data was compared with SGT data at the same elution time (#, $p < 0.05$).

3.2. Interaction between sAA Level and LCGT Intake

Among the 20 initial participants, one abstained due to poor physical conditions. Nineteen participants drank SGT or LCGT every day from Saturday to Friday. During working days, the participants measured their own level of sAA every morning and evening—i.e., before work and after work, respectively—and, in the evening, recorded the volume consumed throughout the day. The mean levels of sAA in all participants were compared between the SGT and LCGT groups. The level of sAA after work was significantly lower when participants consumed LCGT than when they consumed SGT (Figure 2a, $p = 0.043$). Similarly, the level of sAA before work tended to be lower in the LCGT group than in the SGT group ($p = 0.109$).

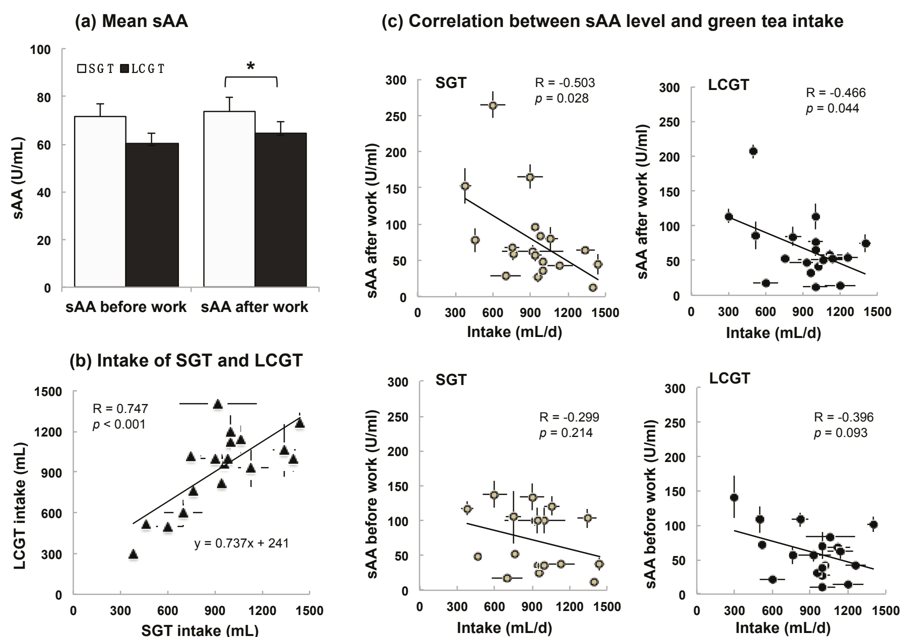


Figure 2. Anti-stress effect of LCGT. (**a**) Mean sAA level of each group. Data are expressed as mean ± SEM (n = 19, *, $p < 0.05$; one-way ANOVA); (**b**) Correlation between SGT and LCGT intake; (**c**) Correlation between the level of sAA before work or after work and SGT or LCGT intake. Data of b and c are expressed as mean ± SEM (n = 5, in each participant).

The mean volumes consumed by each participant were 929 ± 31 mL of SGT and 927 ± 34 mL LCGT. LCGT intake was closely correlated with SGT intake in each participant (Figure 2b, $p < 0.001$). Then, the interaction between sAA level and ingestion volume was examined. Ingestion volume was negatively and significantly correlated with the level of sAA after work (Figure 2c, SGT, $p = 0.028$; LCGT, $p = 0.044$). The correlation between ingestion volume and the levels of sAA before work was low (Figure 2c, SGT, $p = 0.214$; LCGT, $p = 0.093$).

Although there were individual differences in the levels of sAA, participants with higher sAA before work showed higher sAA after work in both SGT and LCGT groups (Figure 3a, SGT $p = 0.002$, LCGT $p < 0.001$). There was no significant difference in the slope of the approximate line (SGT 0.935, LCGT 0.897). Then, the effect of SGT and LCGT intake was compared on the level of sAA in each participant. The levels of sAA became low in each participant by changing from SGT to LCGT (Figure 3b, the slope of the approximate line; sAA before work 0.640, sAA after work 0.710).

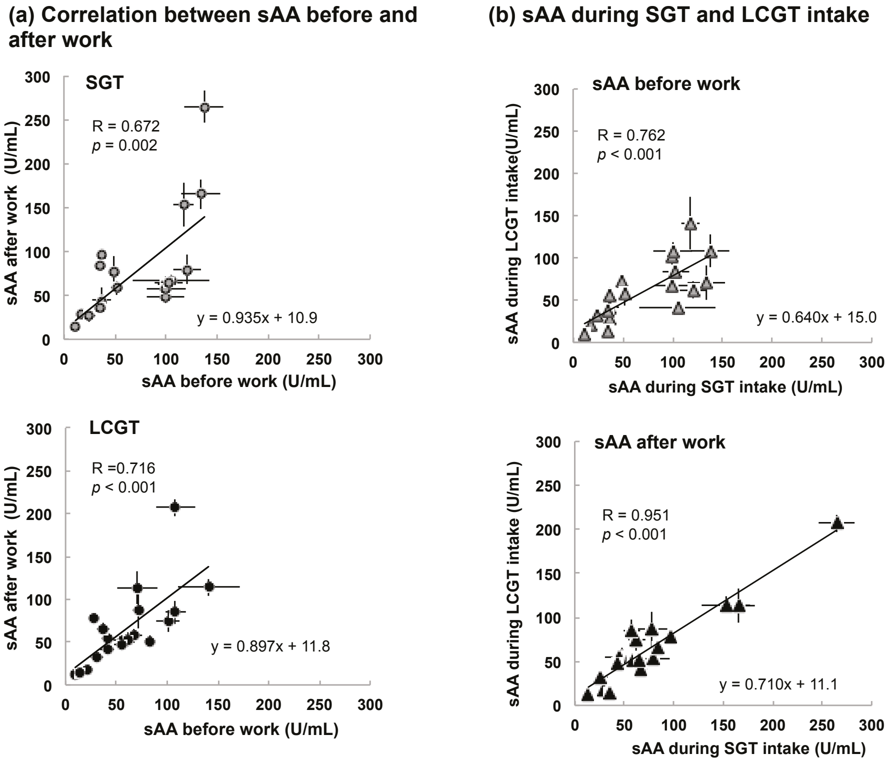


Figure 3. Level of sAA in each participant. (**a**) Correlation between sAA before and after work; (**b**) Correlation of sAA before or after work between during SGT and LCGT intake. Data are expressed as mean ± SEM (n = 5, in each participant).

3.3. Effect of SGT and LCGT Ingestion on Sleep Parameters

Sleep data of the participants was obtained using a 1ch EEG system. Since there was no difference in sleep time of each participant in the period of EEG measurements, data from the second day was used for the analysis. The data of sleep parameters when each participant drank SGT or LCGT are shown in Table 2. Mean values of SL, REM SL, SPT, TST, REM, N1, N2+N3, WASO, AVR SLC, SE, and 1st δ did not differ significantly between SGT and LCGT. However, the time of B2 WASO tended to be shorter when participants ingested LCGT than SGT (Table 2 and Figure 4a, p = 0.065). The level of sAA before work was closely correlated with SPT and TST in participants that ingested SGT (Table 2 and Figure 4b, p = 0.046 and 0.036, respectively). There was no correlation between TST and SGT intake (Figure 4c). The level of N2+N3 tended to be negatively correlated with sAA before work in the LCGT group (Figure 4d, p = 0.080). A positive correlation was observed between intake volumes of LCGT and the level of N2+N3 (Figure 4e, p = 0.045). There was no significant relationship between sAA after work and sleep parameters (Table 2).

Table 2. Mean value of each sleep parameter and the correlation between sleep parameters and sAA or intake volume when participants drank SGT or LCGT.

Sleep Parameters	Abbreviation	Standard Green Tea							Low-Caffeine Green Tea						
		Mean ± SEM	Correlation between sAA before Work		Correlation between sAA after Work		Correlation between Intake Volume		Mean ± SEM	Correlation between sAA before Work		Correlation between sAA after Work		Correlation between Intake Volume	
			R	*p* Value	R	*p* Value	R	*p* Value		R	*p* Value	R	*p* Value	R	*p* Value
Onset of sleep (min)	SL	12.6 ± 3.0	−0.129	0.599	−0.239	0.325	0.356	0.135	15.7 ± 4.9	0.075	0.759	−0.026	0.915	−0.138	0.572
Onset of REM (min)	REM SL	63.5 ± 5.3	0.184	0.450	−0.199	0.415	0.427	0.068	57.7 ± 3.0	0.045	0.854	−0.045	0.854	0.304	0.207
Total period of sleep (min)	SPT	351.3 ± 12.5	0.462	0.046 *	0.232	0.339	−0.136	0.579	350.2 ± 14.6	0.173	0.479	0.024	0.921	0.301	0.211
Total sleep time (SPT−WASO) (min)	TST	328.7 ± 12.7	0.484	0.036 *	0.2672	0.269	−0.138	0.572	328.6 ± 13.5	0.130	0.595	0.040	0.872	0.309	0.198
Rapid eye movement (%)	REM	28.2 ± 2.5	0.066	0.787	−0.082	0.739	−0.106	0.665	25.9 ± 1.2	0.077	0.753	0.142	0.562	−0.186	0.446
Light sleep (%)	N1	8.7 ± 0.7	0.361	0.129	0.2039	0.402	−0.055	0.822	9.3 ± 0.8	0.285	0.236	0.375	0.113	−0.438	0.061
Slow wave sleep (%)	N2+N3	58.8 ± 1.4	0.197	0.420	0.1261	0.607	−0.137	0.577	58.7 ± 1.2	−0.411	0.080	−0.351	0.141	0.464	0.045 *
Time spent awake during the sleep (min)	WASO	22.6 ± 2.9	−0.117	0.635	−0.163	0.505	0.018	0.943	21.7 ± 2.3	0.329	0.169	−0.076	0.757	0.096	0.697
Total awakening time during two hours before the final awakening (min)	B2 WASO	18.5 ± 4.4	0.009	0.969	−0.219	0.367	−0.126	0.607	9.7 ± 1.5	−0.018	0.941	−0.139	0.571	0.250	0.301
Average sleep cycle (min)	AVR SLC	91.5 ± 3.2	−0.032	0.895	−0.268	0.267	−0.189	0.437	90.4 ± 2.9	0.006	0.979	−0.248	0.306	0.176	0.471
Sleep eficiency (%)	SE	88.5 ± 1.7	0.099	0.688	0.281	0.244	−0.122	0.620	89.2 ± 1.5	−0.153	0.533	0.055	0.824	0.110	0.654
δ power in the 1st sleep cycle (μV^2)	1st δ	149,256 ± 18,656	−0.031	0.900	−0.157	0.520	−0.052	0.832	120644 ± 15923	−0.019	0.938	0.158	0.518	−0.254	0.295
sAA (U/mL)			71.7 ± 5.2		73.9 ± 5.9					60.5 ± 4.3		64.7 ± 4.8			
Intake volume (mL)							929 ± 31							927 ± 34	

Data of sleep parameters are expressed as mean ± SEM (n = 19). Correlation coefficient (R) and p value between each sleep parameter and sAA or intake volume of each participant are expressed (*, $p < 0.05$).

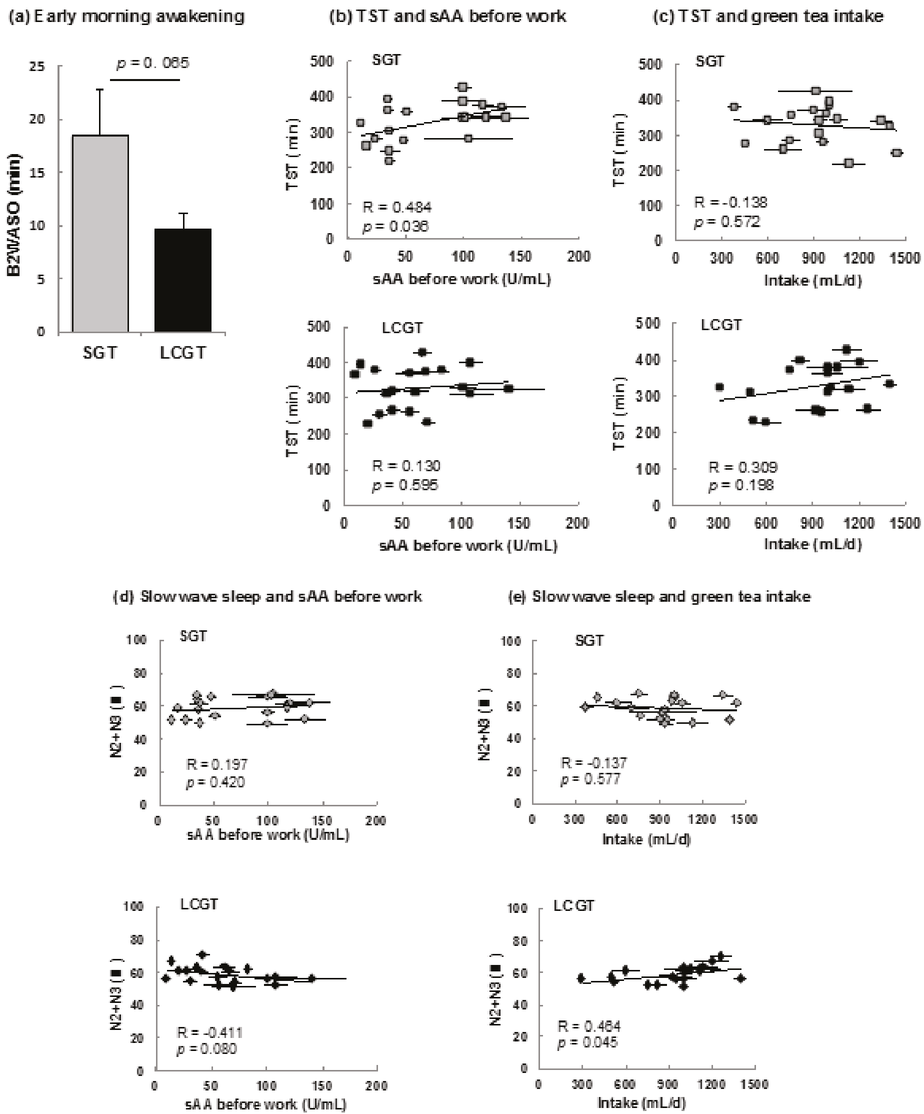


Figure 4. Effect of green tea intake on sleep parameters or sAA. (**a**) Early morning awakening. Data are expressed as mean ± SEM (n = 19, *, p = 0.065; one-way ANOVA); (**b**) Correlation between TST and sAA before work; (**c**) Correlation between TST and intake volume; (**d**) Correlation between slow wave sleep (N2+N3) and sAA before work; (**e**) Correlation between slow wave sleep and intake of SGT or LCGT. Data of b–e are expressed as mean ± SEM (n = 5, in each participant).

3.4. Subjective Stress and Fatigue

Subjective stress tended to be lower when participants drank LCGT than SGT (Figure 5a, p = 0.152), although the physical condition between LCGT and SGT groups was not different (Figure 5b, p = 0.779). The accumulated fatigue that participants felt on Monday morning was significantly lower when they drank LCGT (Figure 5c, p = 0.015). Subjective fatigue on Friday evening tended to be lower in the

LCGT group than the SGT group (Figure 5c, $p = 0.074$). The evaluation of work severity was similar when participants drank LCGT or SGT (Figure 4d, Monday $p = 0.316$, Friday $p = 0.531$).

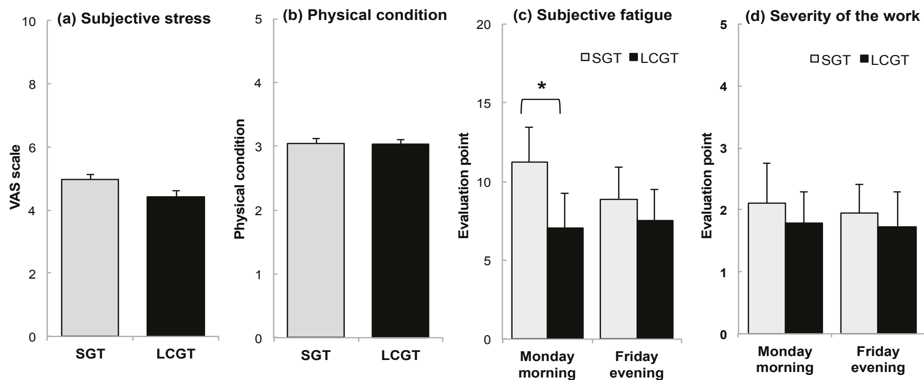


Figure 5. Effect of green tea ingestion on subjective stress and fatigue. (**a**) Subjective stress; (**b**) physical condition; (**c**) subjective fatigue; and (**d**) severity of work. Data are expressed as mean ± SEM ($n = 19$, *, $p < 0.05$; one-way ANOVA).

4. Discussion

The level of sAA after work was significantly lower in the LCGT group than in the SGT group (Figure 2a). The main difference between LCGT and SGT was the amount of caffeine (Figure 1a). Caffeine intake was ~1 mg/kg in those participants who ingested LCGT. Low dose of caffeine (0.32 mg/kg) did not suppress stress response in mice, but was canceled by theanine [11]. In addition, EGCG suppressed the anti-stress effect of theanine [11]. Thus, low doses of caffeine are not beneficial, but suggest that the interaction among theanine, caffeine, and EGCG is important for suppression of stress. In SGT, the ratio of theanine: caffeine: EGCG was 1:1:0.5 while in LCGT, it was 1:0.3:0.3. Since the ratio of theanine to caffeine and EGCG was 2.5 times higher in LCGT than in SGT, the anti-stress effect of theanine was considered to be higher in LCGT than SGT.

Participants that ingested <600 mL of LCGC exhibited a higher level of sAA than the mean (64.7 U/mL) (Figure 2c). That is, intake of at least 600 mL of LCGT may be required to suppress sAA. When LCGT was eluted with room temperature water, in the case of 3 h elution, the concentration of theanine was 100 mg/L (Figure 1c). It can be extrapolated that at least 60 mg/day of theanine may be needed to suppress stress. A similar anti-stress effect had been observed in elderly participants that ingested ≥60 mg/day of theanine from LCGT [28].

Whereas there was no significant difference in the mean value of various sleep parameters between SGT and LCGT, the early morning awakening time tended to be shorter by changing from SGT to LCGT. Early morning awakening is a frequent complaint among patients with sleep disorders [29,30]. The ratio of non-REM sleep (N2+N3), a marker of deep sleep, was significantly higher in those participants that consumed a higher amount of LCGT. Increased theanine intake may increase sleep quality. The time of SPT and TST was closely correlated with sAA before work rather than after work, suggesting that sAA after work does not influence sleep, but sleep affects sAA before work. Participants of high sAA before work may have required a longer sleeping time. Indeed, the average sleeping time of participants was short (5.5 h).

Subjective fatigue is reported to be higher on Monday than on Friday [31]. It is worth noting that subjective fatigue was significantly reduced by LCGT intake. High subjective fatigue on Monday is greatly affected by weekend rest and lack of motivation at the beginning of the week [31]. The levels of sAA were not measured on the weekend; however, the ingestion of green tea was initiated from

Saturday. Therefore, participants in the LCGT group are presumed to have lower sAA than members of the SGT group.

In workers who habitually have a short sleep pattern (mean weekday sleep ≤6 h), the extension of sleep on weekends improves alertness and performance during the first few days in subsequent weeks [32]. Strategic naps may reduce subjective feelings of fatigue and improve performance and alertness [33]. The participants in this study had a habitually short amount of sleep, but sleep extension on the weekend and naps were not observed (data not shown). This suggests that the suppression of stress on the weekend is important for adequate rest on the weekend and to stimulate high motivation at the beginning of the week.

We aimed to explore the effect of green tea with reduced caffeine content on the stress of middle-aged individuals. However, this study has several limitations. Firstly, this study has a small number of participants. However, the sAA analyses using individual differences complemented the small number of participants. Secondly, SGT was used as a control beverage, because water was not a suitable placebo in the blind test. Since participants drank green tea daily, to withdraw the effect of caffeine, a seven-day washout period was set before the test term [34]. Then, intake of LCGT was compared with that of SGT. Thirdly, we used a single-channel EEG instead of gold–standard polysomnography (PLG) to assess sleep. However, it has recently been shown that a single-channel EEG can also be a useful research tool in assessing REM, non-REM sleep and several other parameters [35]. Fourthly, we did not measure the components of all the tea samples that were prepared daily by participants. One possible problem is that the concentration of tea components among participants may have been different. However, the eluate data indicates that the ratio of catechins, caffeine, and amino acids was almost the same in SGT or LCGT (Figure 1), suggesting that the content of tea components was similar under the same elution conditions, including the same volume of tea leaves and water, and a similar temperature. Therefore, despite these limitations, the anti-stress effect of green tea obtained in this experiment is considered to be reliable.

5. Conclusions

The effects of LCGT on stress and sleep were examined in middle–aged individuals (n = 19, mean age 51.3 ± 6.7 years) in a double-blind crossover design using SGT as the control beverage. The level of sAA, a maker of stress, was significantly lower in the participants that consumed LCGT than SGT. Furthermore, ingestion of LCGT significantly improved sleep quality and reduced subjective fatigue on Monday morning. These results suggest that a reduction of caffeine in green tea is beneficial for reducing stress. Simultaneously with the lowering of caffeine, the increase in theanine and Arg are considered to be necessary, it is necessary to further clarify the mechanism of action of the stress-reducing effect of green tea components.

Acknowledgments: The authors thank all participants of the study. This research study was supported by a Grant-in-Aid for Scientific Research (KAKENHI 15K00828) and a grant for specially promoted research of the University of Shizuoka.

Author Contributions: K.U., H.Y. and Y.N. designed the experiment. S.N., Y.K., A.M. and K.I. conducted data analysis. All authors contributed to data interpretation. K.U. drafted the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Grove, K.A.; Lambert, J.D. Laboratory, epidemiological, and human intervention studies show that tea (*Camellia sinensis*) may be useful in the prevention of obesity. *J. Nutr.* **2010**, *140*, 446–453. [CrossRef] [PubMed]
2. Bell, L.; Lamport, D.J.; Butler, L.T.; Williams, C.M. A review of the cognitive effects observed in humans following acute supplementation with flavonoids, and their associated mechanisms of action. *Nutrients* **2015**, *7*, 10290–10306. [CrossRef] [PubMed]

3. Horie, H.; Kohata, K. General ingredients of tea. In *Health Science of Tea, New Possibility for Physiological Function*; Muramatsu, K., Oguni, I., Isemura, M., Sugiyama, K., Yamamoto-Maeda, M., Eds.; Gakkai Shuppan Center: Tokyo, Japan, 2002; pp. 26–31.
4. Unno, K.; Fujitani, K.; Takamori, N.; Takabayashi, F.; Maeda, K.; Miyazaki, H.; Tanida, N.; Iguchi, K.; Shimoi, K.; Hoshino, M. Theanine intake improves the shortened lifespan, cognitive dysfunction and behavioural depression that are induced by chronic psychosocial stress in mice. *Free Radic. Res.* **2011**, *45*, 966–974. [CrossRef] [PubMed]
5. Kimura, K.; Ozeki, M.; Juneja, L.R.; Ohira, H. L-Theanine reduces psychological and physiological stress responses. *Biol. Psychol.* **2007**, *74*, 39–45. [CrossRef] [PubMed]
6. Unno, K.; Iguchi, K.; Tanida, N.; Fujitani, K.; Takamori, N.; Yamamoto, H.; Ishii, N.; Nagano, H.; Nagashima, T.; Hara, A.; et al. Ingestion of theanine, an amino acid in tea, suppresses psychosocial stress in mice. *Exp. Physiol.* **2013**, *98*, 290–303. [CrossRef] [PubMed]
7. Unno, K.; Tanida, N.; Ishii, N.; Yamamoto, H.; Iguchi, K.; Hoshino, M.; Takeda, A.; Ozawa, H.; Ohkubo, T.; Juneja, L.R.; et al. Anti-stress effect of theanine on students during pharmacy practice: Positive correlation among salivary α-amylase activity, trait anxiety and subjective stress. *Pharmacol. Biochem. Behav.* **2013**, *111*, 128–135. [CrossRef] [PubMed]
8. Kakuda, T. Neuroprotective effects of theanine and its preventive effects on cognitive dysfunction. *Pharmacol. Res.* **2011**, *64*, 162–168. [CrossRef] [PubMed]
9. Inoue, K.; Miyazaki, Y.; Unno, K.; Min, J.Z.; Todoroki, K.; Toyo'oka, T. Stable isotope dilution HILIC-MS/MS method for accurate quantification of glutamic acid, glutamine, pyroglutamic acid, GABA and theanine in mouse brain tissues. *Biomed. Chromatogr.* **2016**, *30*, 55–61. [CrossRef] [PubMed]
10. Mehta, A.; Prabhakar, M.; Kumar, P.; Deshmukh, R.; Sharma, P.L. Excitotoxicity: Bridge to various triggers in neurodegenerative disorders. *Eur. J. Pharmacol.* **2013**, *698*, 6–18. [CrossRef] [PubMed]
11. Unno, K.; Hara, A.; Nakagawa, A.; Iguchi, K.; Ohshio, M.; Morita, A.; Nakamura, Y. Anti-stress effects of drinking green tea with lowered caffeine and enriched theanine, epigallocatechin and arginine. *Phytomedicine* **2015**, *23*, 1365–1374. [CrossRef] [PubMed]
12. Unno, K.; Yamada, H.; Iguchi, K.; Ishida, H.; Iwao, Y.; Morita, A.; Nakamura, Y. Anti-stress effect of green tea with lowered caffeine on human: A pilot study. *Biol. Pharm. Bull.* **2017**, *40*, 902–909. [CrossRef] [PubMed]
13. Shimotoku, T.; Ichikawa, H.; Anan, T.; Takayanagi, H.; Ikegaya, K. Relation between amounts of some ingredients extracted from green tea and brewing conditions. *Tea Res. J. (Chagyo Kenkyu Hokoku)* **1982**, *55*, 43–50. [CrossRef]
14. Monobe, M.; Ema, K.; Tokuda, Y.; Maeda-Yamamoto, M. Effect on the epigallocatechin gallate/epigallocatechin ratio in a green tea (*Camellia sinensis* L.) extract of different extraction temperatures and its effect on IgA production in mice. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 2501–2503. [CrossRef] [PubMed]
15. Nater, U.M.; Rohleder, N. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: Current state of research. *Psychoneuroendocrinology* **2009**, *34*, 486–496. [CrossRef] [PubMed]
16. Almela, M.; Hidalgo, V.; Villada, C.; van der Meij, L.; Espín, L.; Gómez-Amor, J. Salvador A. Salivary alpha-amylase response to acute psychosocial stress: The impact of age. *Biol. Psychol.* **2011**, *87*, 421–429. [CrossRef] [PubMed]
17. Nater, U.M.; Rohleder, N.; Schlotz, W.; Ehlert, U.; Kirschbaum, C. Determinants of the diurnal course of salivary alpha-amylase. *Psychoneuroendocrinology* **2007**, *32*, 392–401. [CrossRef] [PubMed]
18. Nater, U.M.; La Marca, R.; Florin, L.; Moses, A.; Langhans, W.; Koller, M.M.; Ehlert, U. Stress-induced changes in human salivary alpha-amylase activity–associations with adrenergic activity. *Psychoneuroendocrinology* **2006**, *31*, 49–58. [CrossRef] [PubMed]
19. Klein, L.C.; Bennett, J.M.; Whetzel, C.A.; Granger, D.A.; Ritter, F.E. Caffeine and stress alter salivary alpha-amylase activity in young men. *Hum. Psychopharmacol.* **2010**, *25*, 359–367. [CrossRef] [PubMed]
20. Castro-Diehl, C.; Diez Roux, A.V.; Redline, S.; Seeman, T.; Shrager, S.E.; Shea, S. Association of sleep duration and quality with alterations in the hypothalamic-pituitary adrenocortical axis: The Multi-Ethnic Study of Atherosclerosis (MESA). *J. Clin. Endocrinol. Metab.* **2015**, *100*, 3149–3158. [CrossRef] [PubMed]
21. Räikkönen, K.; Matthews, K.A.; Pesonen, A.K.; Pyhälä, R.; Paavonen, E.J.; Feldt, K.; Jones, A.; Phillips, D.I.; Seckl, J.R.; Heinonen, K.; et al. Poor sleep and altered hypothalamic-pituitary- adrenocortical and sympatho-adrenal-medullary system activity in children. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 2254–2261. [CrossRef] [PubMed]

22. Horie, H.; Maeda-Yamamoto, M.; Ujihara, T.; Kohata, K. Extraction of tea catechins for chemical analysis. *Tea Res. J. (Chagyo Kenkyu Hokoku)* **2002**, *94*, 60–64. [CrossRef]
23. Goto, T.; Horie, H.; Mukai, T. Analysis of major amino acids in green tea by high-performance liquid chromatography coupled with OPA precolumn derivatization. *Tea Res. J. (Chagyo Kenkyu Hokoku)* **1993**, *77*, 29–33. [CrossRef]
24. Yamaguchi, M.; Kanemori, T.; Kanemaru, M.; Takai, N.; Mizuno, Y.; Yoshida, H. Performance evaluation of salivary amylase activity monitor. *Biosens. Bioelectron.* **2004**, *20*, 491–497. [CrossRef] [PubMed]
25. Yoshida, M.; Kashiwagi, K.; Kadotani, H.; Yamamoto, K.; Koike, S.; Matsuo, M.; Yamada, N.; Okawa, M.; Urade, Y. Validation of a portable single-channel EEG monitoring system. *J. Oral Sleep Med.* **2015**, *1*, 140–147.
26. Yoshida, M.; Shinohara, H.; Kodama, H. Assessment of nocturnal sleep architecture by actigraphy and one-channel electroencephalography in early infancy. *Early Hum. Dev.* **2015**, *91*, 519–526. [CrossRef] [PubMed]
27. Flexer, A.; Gruber, G.; Dorffner, G. A reliable probabilistic sleep stager based on a single EEG signal. *Artif. Intell. Med.* **2005**, *33*, 199–207. [CrossRef] [PubMed]
28. Unno, K.; Noda, S.; Kawasaki, Y.; Yamada, H.; Morita, A.; Iguchi, K.; Nakamura, Y. Ingestion of green tea with lowered caffeine improves sleep quality of the elderly via suppression of stress. *J. Clin. Biol. Nutr.* **2017**, *61*, 1–7.
29. Lui, S.L.; Ng, F.; Lo, W.K. Factors associated with sleep disorders in Chinese patients on continuous ambulatory peritoneal dialysis. *Perit Dial Int.* **2002**, *22*, 677–682. [PubMed]
30. Janson, C.; Gislason, T.; De Backer, W.; Plaschke, P.; Björnsson, E.; Hetta, J.; Kristbjarnason, H.; Vermeire, P.; Boman, G. Prevalence of sleep disturbances among young adults in three European countries. *Sleep* **1995**, *18*, 589–597. [PubMed]
31. Kobayashi, H.; Demura, S.; Goshi, F.; Minami, M.; Nagasawa, Y.; Sato, S. Trends in variation within the day and between days of subjective symptoms of fatigue in adolescent males. *Nihon Eiseigaku Zasshi* **2000**, *54*, 622–630. [CrossRef] [PubMed]
32. Kubo, T.; Takahashi, M.; Sato, T.; Sasaki, T.; Oka, T.; Iwasaki, K. Weekend sleep intervention for workers with habitually short sleep periods. *Scand. J. Work Environ. Health* **2011**, *37*, 418–426. [CrossRef] [PubMed]
33. Hartzler, B.M. Fatigue on the flight deck: The consequences of sleep loss and the benefits of napping. *Accid. Anal. Prev.* **2014**, *62*, 309–318. [CrossRef] [PubMed]
34. Griffiths, R.R.; Woodson, P.P. Caffeine physical dependence: A review of human and laboratory animal studies. *Psychopharmacology (Berl.)* **1988**, *94*, 437–451. [CrossRef] [PubMed]
35. Lucey, B.P.; Mcleland, J.S.; Toedebusch, C.D.; Boyd, J.; Morris, J.C.; Landsness, E.C.; Yamada, K.; Holtzman, D.M. Comparison of a single-channel EEG sleep study to polysomnography. *J. Sleep Res.* **2016**, *25*, 625–635. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Case Report

Synergistic Interplay between Curcumin and Polyphenol-Rich Foods in the Mediterranean Diet: Therapeutic Prospects for Neurofibromatosis 1 Patients

Teresa Esposito [1], Carla Schettino [2], Paola Polverino [2], Salvatore Allocca [1], Laura Adelfi [1], Alessandra D'Amico [3], Guglielmo Capaldo [2], Bruno Varriale [4], Anna Di Salle [5], Gianfranco Peluso [5], Giuseppe Sorrentino [6], Giacomo Lus [2], Simone Sampaolo [2], Giuseppe Di Iorio [2] and Mariarosa Anna Beatrice Melone [2,*]

1. Department of Experimental Medicine, Section of Human Physiology and Unit of Dietetics and Sports Medicine, University of Campania Luigi Vanvitelli, via Santa Maria di Costantinopoli 16, 80138 Naples, Italy; teresa.esposito@unicampania.it (T.E.); alloccasalvatore2016@libero.it (S.A.); laura.adelfi@hotmail.it (L.A.)
2. Division of Neurology, Center for Rare Diseases & Inter University Center for Research in Neurosciences, Department of Medical, Surgical, Neurologic, Metabolic and Aging Sciences, University of Campania Luigi Vanvitelli, via Sergio Pansini, 5, 80131 Naples, Italy; carla.schettino@unicampania.it (C.S.); polverinopaola@gmail.com (P.P.); dr.guglielmo.capaldo@virgilio.it (G.C.); giacomo.lus@unicampania.it (G.L.); simone.sampaolo@unicampania.it (S.S.); giuseppe.diiorio@unicampania.it (G.D.I.)
3. Department of Advanced Biomedical Sciences, Neuroradiology, University "Federico II", via Pansini, 5, 80131 Naples, Italy; damicoalex@tiscali.it
4. Department of Experimental Medicine, Laboratory of Molecular Biology and Genetics, University of Campania Luigi Vanvitelli, via Santa Maria di Costantinopoli 16, 80138 Naples, Italy; bruno.varriale@unicampania.it
5. Institute of Agro-environmental and Forest Biology, National Research Council, via Pietro Castellino 111, 80131 Naples, Italy; anna.disalle@cnr.it (A.D.S.); gianfranco.peluso@cnr.it (G.P.)
6. ISAFOM, National Research Council, via Patacca 85, Ercolano (NA), 80056 Naples, Italy; giuseppe.sorrentino@isafom.cnr.it

* Correspondence: marina.melone@unicampania.it; Tel.: +39-0815666810

Received: 29 June 2017; Accepted: 18 July 2017; Published: 21 July 2017

Abstract: Neurofibromas are the hallmark lesions in Neurofibromatosis 1 (NF1); these tumors are classified as cutaneous, subcutaneous and plexiform. In contrast to cutaneous and subcutaneous neurofibromas, plexiform neurofibromas can grow quickly and progress to malignancy. Curcumin, a turmeric-derived polyphenol, has been shown to interact with several molecular targets implicated in carcinogenesis. Here, we describe the impact of different dietary patterns, namely Mediterranean diet (MedDiet) compared to the Western diet (WesDiet), both with or without curcumin, on NF1 patients' health. After six months, patients adopting a traditional MedDiet enriched with 1200 mg curcumin per day (MedDietCurcumin) presented a significant reduction in the number and volume of cutaneous neurofibromas; these results were confirmed in subsequent evaluations. Notably, in one patient, a large cranial plexiform neurofibroma exhibited a reduction in volume (28%) confirmed by Magnetic Resonance Imaging. Conversely, neither unenriched MedDiet nor WesDiet enriched with curcumin exhibited any significant positive effect. We hypothesize that the combination of a polyphenol-rich Mediterranean diet and curcumin was responsible for the beneficial effect observed on NF1. This is, to the best of our knowledge, the first experience with curcumin supplementation in NF1 patients. Our report suggests that an integrated nutritional approach may effectively aid in the management of NF1.

Keywords: neurofibromatosis 1; neurofibromas; curcumin; diet; polyphenols

1. Introduction

Polyphenols are secondary metabolites ubiquitous in the plant kingdom with unique antioxidant properties and a wide spectrum of therapeutic activities. It is well known that the phenolic compounds present mainly in olive oil contribute significantly to the superior health profile observed in Mediterranean populations following the traditional Mediterranean diet (MedDiet). In addition, curcumin, the most bioactive polyphenolic constituent of turmeric and an integral part of the Indian diet, exerts protective effects on a variety of diseases such as cardiac diseases, cancer, diabetes, Alzheimer's disease, rheumatoid arthritis and psoriasis [1–6].

However, the mechanisms by which dietary polyphenols can affect human diseases are multiple and complex. Various stages of carcinogenesis may be inhibited by polyphenols in in vitro or in vivo systems. For example, curcumin is able to inhibit diethylnitrosamine-induced hepatocarcinogenesis in mice at a concentration of 0.2% in the diet [7], but the biological mechanism(s) of this effect has only been partially clarified. Again, olive oil polyphenols seem to exert anticancer effects through the modulation of genes and molecular signaling pathways associated with cell survival, cell cycle progression, cell growth arrest and apoptosis, as demonstrated in several tumor cell lines [8]. Recent findings suggest that polyphenols may exert anti-proliferative activity by affecting the cell metabolism. In particular, polyphenols have the potential to modulate glucose uptake, and to alter the glutathione as well as lipid metabolism [9]. Furthermore, there is experimental evidence that supports an effect of polyphenols on the entry of glutamine, an amino-acid essential for cancer metabolism, into the cells [10].

Interestingly, it has also been reported that polyphenols can affect tumor progression by interfering with the dynamic interactions between several components within the tumor microenvironment and the cancer cells. Indeed, the bi-directional interaction between cancer cells and the surrounding cells increases tumor proliferation, invasion and metastasis, and allows the tumor cells to resist therapeutic insults [11]. Curcumin is able to interfere with the cross-talk between cancer stem cells and stromal fibroblasts, resulting in the reversal of epithelial to mesenchymal transition and associated metastasis [11]. Furthermore, curcumin, by a dual mode of action, is able to modulate the enzymatic activity of the EGF receptor (EGF-R) intracellular domain [12].

In our study, we evaluated the effects of a traditional Mediterranean diet (rich in polyphenol content) compared to a Western dietary "pattern", both with or without curcumin supplementation, in case series of patients with Neurofibromatosis type 1 (NF1), one of the most common autosomal dominant genetic disorders that affects ~1 in 3500 individuals [13]. The NF1 gene (17q11.2.5; NM_000267.3) encodes neurofibromin, a cytoplasmic protein of 2818 amino acids (molecular mass of 220–250 kDa) that promotes the intrinsic GTPase activity of Ras [14]. Nearly all NF1 patients develop dermal neurofibromas. In addition, they can develop brain tumors (gliomas and glioblastomas) and peripheral nerve tumors (spinal neurofibromas, plexiform neurofibromas and malignant peripheral nerve sheath tumors). The NF1 phenotype is not complete at birth despite being a genetic disease [13,14]. The mutation of NF1 has full penetrance and the symptoms appear in an age-dependent manner [13,14]. Therefore, the influence of nutrition is fundamental in the epigenetic control of the disease. Of interest, de Souza et al, reporting that NF1 patients consumed an unhealthy diet, rich in fats and sodium and poor in fiber, vitamins, and minerals, hypothesized a role of the dietary and nutritional patterns in the severity of the clinical manifestations of NF1 [15]. In addition, Carotenuto and Esposito demonstrated that adjustments of the diet influenced the clinical features in NF1. In particular, the addition of nutraceuticals improved several symptoms affecting NF1 patients, such as migraine-related disability [16]. Although considerable progress has been made in understanding NF1, no effective therapy is available to treat this pathological condition. Herein

we demonstrated that a Mediterranean diet and curcumin act synergically to induce a significant reduction in the number and size of neurofibromas, thus suggesting that an integrated nutritional approach could be effective in the management of NF1.

2. Materials and Methods

Our series included eleven NF1 patients identified on the basis of the National Institutes of Health (NIH) Consensus Conference criteria (National Institutes of Health Consensus Development Conference Statement: Neurofibromatosis 1988), clinically followed up at our Division of Neurology, Neurofibromatosis and Rare Diseases Center of the University Hospital, University of Campania Luigi Vanvitelli, Italy (Table 1). Of these eleven patients, seven lacked a family history of NF1.

Eight patients were screened for NF1 mutations (see Table 1). Genetic testing revealed that six of them carried a mutation producing truncated neurofibromin, as confirmed by a new diagnostic technique recently described by our group [17]; Patient 9 presented a single-nucleotide mutation, while Patient 3 presented a newly discovered intragenic heterozygous deletion encompassing exon 12 and 13 [18].

2.1. Methods

The NF1 patients were divided into four groups on the basis of their dietary regimen: the first group (n = 2) was instructed to follow a Western diet (WesDiet); the second (n = 3) a traditional Mediterranean-style diet (MedDiet); the third (n = 3) a Western diet enriched with curcumin (WesDietCurcumin); and the fourth (n = 3) a traditional Mediterranean-style diet enriched with curcumin (MedDietCurcumin). All the diets were evaluated by a dietitian. In particular, the Western diet included ad libitum the consumption of red and processed meat, refined grains, French fries, sweets and desserts. No total calorie restriction was advised.

The traditional Mediterranean-style diet included the abundant use of olive oil for cooking and dressing dishes (extra-virgin olive oil—50 mL/day with >2000 mg/kg total phenol concentration as determined by Folin-Ciocalteu's assay—Sigma Aldrich, St. Louis, MO, USA), the consumption of ≥2 daily servings of vegetables, ≥3 daily servings of fresh fruits, ≥3 weekly servings of legumes, ≥3 weekly servings of fish or seafood, white meats instead of red meats or processed meats (burgers, sausages), and avoiding the consumption of cream, butter, margarine, pate, duck, carbonated and/or sugared beverages, pastries, industrial bakery products and desserts, French fries or potato chips, and sweets. Curcumin was supplemented at a dose of 1200 mg to the third and fourth groups at lunchtime [19,20]. Finally, all patients were invited to restrict foods known to contain curcumin.

Physical activity was not specifically advised for all NF1 patients. Compliance with the program was assessed by attendance at the follow-up visits and completion of the diet diaries. The patients were seen at two-monthly intervals over the six months of follow-up, after the introduction of the different diets. A complete physical examination, an evaluation of plasmatic curcumin level, integrated by photographic documentation were performed at each visit. Manual counting one by one of cutaneous neurofibromas was performed on all images (to facilitate the counting process, images were re-elaborated using Photoshop® software by Adobe Systems Software, Belfast, Ireland).

Table 1. NF1 patient data.

Diet	Patient ID	Age	Sex	Familiar NF1	Sporadic NF1	Café-Au-Lait Spots Number > 6	Cutaneous or Subcutaneous Neurofibromas	Freckling	Lisch Nodules	Plexiform Neurofibroma	Scoliosis	Optic Pathway Glioma	Molecular Testing	Short Stature	Pathological Findings on Brain MRI
WesDiet	1 A.S.	38	M	yes	no	yes	yes	yes	yes	no	yes	yes	yes	no	T2-Weighted Hyperintensities (Ubo's)
	2 A.F.	22	M	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	no	T2-Weighted Hyperintensities (Ubo's)
MedDiet	3 E.M.	27	M	yes	no	yes	yes	yes	yes	yes	no	no	yes	no	Fusiform aneurysms of both ICAs (ref)
	4 B.E.	18	M	no	yes	yes	yes	yes	yes	yes	yes	no	yes	yes	T2-Weighted Hyperintensities (Ubo's)
	5 D.M.M.	22	M	no	yes	yes	yes	yes	yes	no	yes	no	yes	no	No signs
WesDietCurcumin	6 A.M.C.	41	F	yes	no	yes	yes	yes	yes	no	yes	no	no	no	T2-Weighted Hyperintensities (Ubo's); vascular malformations (hypoplastic mid-distal portion of basilar artery and intra-cranial vertebral artery; intracavernous right carotid aneurysm)
	7 A.N.	59	M	no	yes	yes	yes	yes	yes	yes	yes	no	no	no	No signs
	8 A.F.	44	F	no	yes	yes	yes	yes	yes	no	no	no	no	no	T2-Weighted Hyperintensities (Ubo's); hypoplasia of PCA
MedDietCurcumin	9 P.C.	54	M	no	yes	yes	yes	yes	yes	no	no	no	yes	no	No signs
	10 F.L.	27	F	no	yes	yes	yes	yes	yes	no	no	no	yes	no	No signs
	11 R.S.	50	M	no	yes	yes	yes	yes	yes	yes	yes	no	yes	no	Multiple foci of T2 hyper intensity (vascular gliosis)

Note: Ubo's = unidentified bright objects; ICAs = internal carotid arteries; PCA = posterior cerebral artery; F: Female.

When appropriate, magnetic resonance imaging (MRI) was performed. Head MR was performed using a 1.5 T MRI unit (Philips Gyroscan) including axial Spin-Echo T1, Turbo Spin-Echo (TSE) T2 and FLAIR (slice thickness: 5 mm), coronal FLAIR (slice thickness 3 mm), sagittal TSE T2 (slice thickness: 4 mm) and axial DWI (Diffusion Weighted Imaging). Volumetric analysis was performed using an algorithm for volumetric evaluations available in OsiriX, a software for viewing and processing DICOM images. It was necessary to make a correction of the gap between the slices (intergap correction). The tumor profiles were manually traced "slice by slice".

The patients' nutritional status was evaluated applying a combination of clinical observation, bioimpedentiometric analysis and anthropometric and biochemical parameters.

2.2. Ethics

This study was approved by the Medical Ethics Committee and Safety Board of the University of Campania Luigi Vanvitelli, in accordance with the Declaration of Helsinki on ethical principles for medical research involving human subjects (protocol number 479/13). Written informed consent was obtained from each patient before admission to the study and to allow the initiation of any study-related procedures. In addition, the patients authorized the publication of this manuscript, its accompanying images and other data.

2.3. Plasma Sample Preparation

A plasma aliquot was first treated with 10 µL of 6.0 M HCl and then with 10 U of β-glucuronidase type H-1 from Helix pomatia in 0.1 M phosphate buffer (pH 6.86). The resulting mixture was then thoroughly vortexed and incubated at 37 °C for 1 h to hydrolyze the phase-2 conjugates of curcuminoids. After incubation, curcuminoids were extracted with 2 volumes of methanol/chloroform (1:2 *v*/*v*), sonicated in a water bath for 15 min and evaporated to dryness at 30 °C under negative pressure in a centrifugal concentrator. This process was repeated for a total of two extractions. The dried extract was reconstituted in methanol and subjected to HPLC analysis.

2.4. Chromatographic Analysis of Curcuminoids

The HPLC-UV procedure was conducted according to Heath et al. [21] with some modification. The analysis was carried out on Agilent 1260 Infinity Quaternary LC (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD (Diode-Array Detector). The chromatographic separation was performed on a Gemini® 5 µm C18 110 Å, LC Column 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) protected by a guard column (Security Guard Cartridge C18, 4 × 2.0 mm inner diameter, Phenomenex, Torrance, CA) and maintained at 30 °C. A linear elution gradient consisting of a mobile phase A (0.1% acetic acid), B (Acetonitrile), and C (Methanol) was programmed as follows: initially 50% A, 45% B, and 5% C, linearly changed to 30% A, 65% B, and 5% C over 5 min, and then held for 4 min at 30% A, 65% B, and 5% C. The system was then re-equilibrated for 5 min with the initial solvent. The detection wavelength was set at 420 nm. The quantitation of curcuminoids is by peak area ratio (curcumin, demethoxycurcumin and bisdemethoxycurcumin to internal standard) and is based on a standard curve in a plasma or urine matrix, generated by using an external standard to spike plasma or urine. A linear curve is generated from a single analysis of six different standard concentrations. System control and data acquisition were performed using the ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

2.5. Statistical Analysis

Statistical analysis was conducted using a one-way ANOVA test, with the significant differences determined at $p < 0.05$, using GraphPad Prism Version 5.04 software (GraphPad Software, San Diego, CA, USA).

3. Results

Patients 1, 2, 6, 7 and 8 did not adhere to the MedDiet and did not significantly modify their dietary habits, which were consistent with a WesDiet; among them, Patient 6, 7 and 8 agreed to introduce curcumin to their diet (WesDietCurcumin). Patients 3, 4 and 5 followed a MedDiet. Patients 9, 10 and 11 agreed to follow a MedDietCurcumin. As shown in Table 1, all patients presented a clinical picture characterized by more than six cafè-au-lait spots, intertriginous freckling and both cutaneous and subcutaneous neurofibromas; patients 2, 3, 4, 7 and 11 presented plexiform neurofibromas. NF1 complications were present in patients 1, 2, 4, 5, 6, 7 and 11 with scoliosis, in patient 4 with short stature, and in patients 1 and 2 with optic pathway glioma [22]. Patients 3, 6 and 8 presented cerebrovascular abnormalities, which are a common occurrence in NF1 [23]. In particular, patient 3 presented very rare bilateral aneurysms of both internal carotid arteries, as extensively described in a previous report [18]. Patients 1, 2, 3 and 4 had a family history of NF1.

3.1. Follow-Up

Clinical follow-up integrated by self-assessment of outcome in the first (WesDiet—patients 1 and 2), second (MedDiet—patients 3, 4 and 5) and third group (WesDietCurcumin—patients 6, 7 and 8) did not detect significant phenotypic variations in the number, size and color of cutaneous neurofibromas or in other signs and symptoms of disease, clinically detectable, at the different time points, compared to baseline. Patient 9 (Table 1), a 54-year-old woman, showed a severe cutaneous phenotype. Her medical history was notable for numerous surgical removals of limb and trunk lesions, histologically defined as neurofibromas (Figure 1a–g). A comprehensive physical examination performed at baseline showed widespread cafè-au-lait spots, axillary and inguinal freckling and numerous cutaneous neurofibromas on the neck, trunk, upper and lower limbs and perianal area. These neurofibromas varied in size and shape, with both sessile and pedunculated forms widely represented; they were soft and not painful to the touch. While most smaller neurofibromas were flesh-colored, the area located between the right anterior and posterior axillary lines comprised several tumors with a brownish-red color; a larger, round, pedunculated neurofibroma was particularly prominent (Figure 2a). In this area a manual count of 212 distinct neurofibromas was made (Figure 2b). Six months after the introduction of MedDietCurcumin, as shown in Figure 2c,d, there was a striking reduction in the number of neurofibromas compared to baseline. In fact, at this follow-up appointment, only 110 (51%) neurofibromas were detected (Figure 2f). Besides a decrease in the number of neurofibromas, a decrease in their volume was observed, particularly evident for the larger neurofibromas, which appeared to be considerably smaller in size, lighter in color, and softer to the touch.

Patient 10, (Table 1), a 27-year-old woman diagnosed in infancy due to the presence of more than six cafè-au-lait spots; during adolescence, she developed axillary freckling and several neurofibromas. Prior to the introduction of MedDietCurcumin, she presented diffuse cafè-au-lait spots, bilateral axillary and inguinal freckling and cutaneous neurofibromas of various sizes on the trunk and limbs. Several sessile and pedunculated cutaneous neurofibromas were observed on the left breast, and in particular in the areolar area (Figure 3a); in the area selected, we counted 20 neurofibromas (Figure 3b). At the six-month follow-up, she presented a reduction in the volume of several cutaneous neurofibromas in the area selected. Furthermore, a significant decrease in the number of neurofibromas (30%) was evident, as shown in Figure 3c,d and in the histogram in Figure 3e.

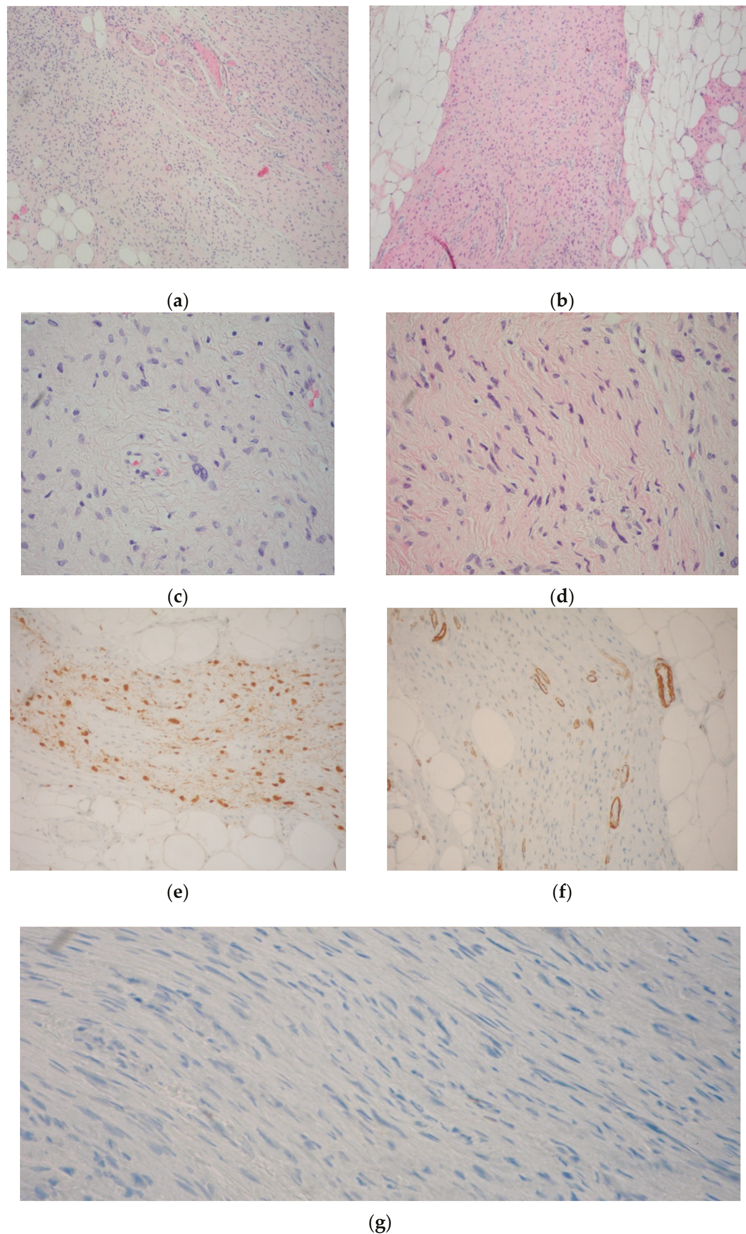


Figure 1. Neurofibroma resection specimen. (**a**,**b**) Spindle "wavy" cells in a matrix of fine fibrillary collagen; neurofibromatous tissue merges with mature fat and ectatic vessels (Hematoxylin-eosin, Magnification 10×); (**c**,**d**) Spindle cell nuclei in a fine fibrillary matrix, at a greater magnification (Hematoxylin-Eosin, Magnification 40×); (**e**) S100 immunohistochemical test confirmed the neurogenic origin of the lesion (Magnification 20×); (**f**) Actin-hhf35 immunohistochemical test excluded the myogenic origin of the lesion (note that only perivascular splindle smooth cells were reactive) (Magnification 20×); (**g**) Proliferation index by Ki67 immunohistochemical test was almost negative (Magnification 20×).

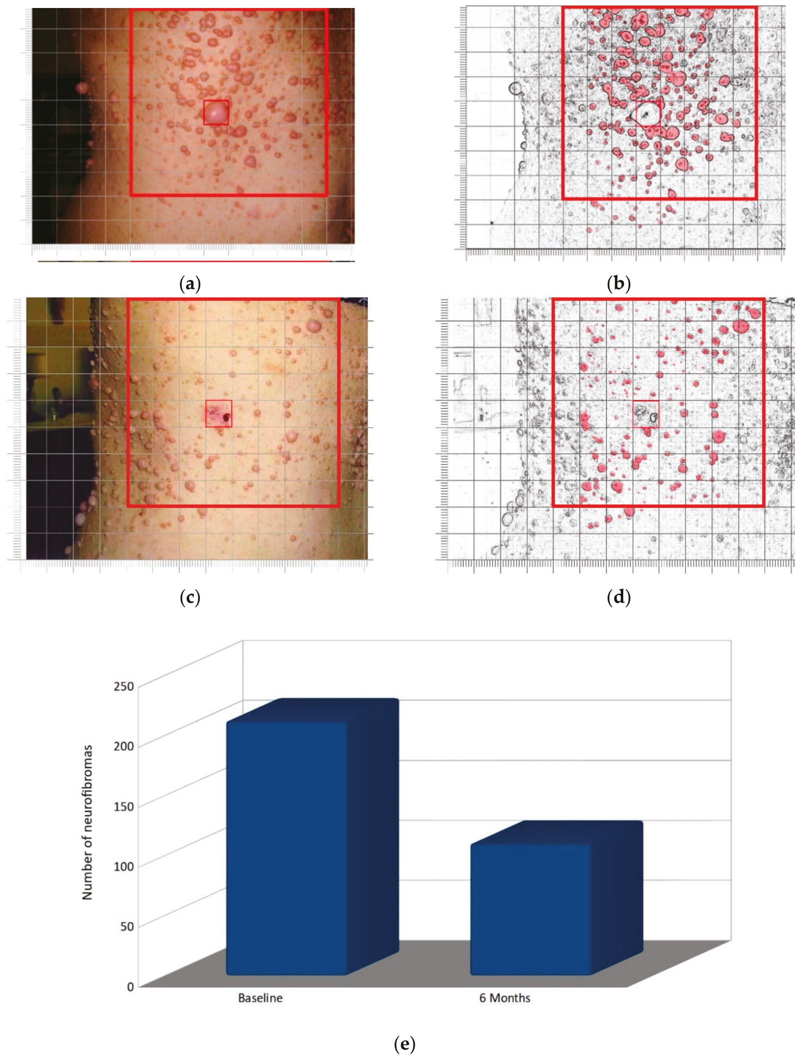


Figure 2. Right lateral view of thoracic region in patient 9: serial clinical assessment. (**a**,**c**) The large red square outlines the sampled area at baseline (**a**) and after six months of MedDietCurcumin (**c**); the small red square indicates the most prominent neurofibroma in the area, which was removed immediately after baseline observation for histological examination; (**b**,**d**) Digital re-elaboration of the images in A and C to facilitate manual counting of cutaneous neurofibromas; (**e**) Bar chart representing the number of neurofibromas in the sampled area at baseline and at six-month follow-up.

Patient 11 (Table 1), a 50-year-old man at the time of our first observation. Since childhood, he presented cafè-au-lait spots and subcutaneous neurofibromas on the trunk and lower limbs; he had also developed a large formation in the left orbito-temporal region, which had been partially resected when he was 44 years old; this lesion had been histologically diagnosed as a plexiform neurofibroma. During the following two years, the residual neurofibroma had increased in volume, infiltrating the ipsilateral orbital cavity and compressing the eyeball and causing partial left palpebral ptosis. Clinical

examination of the lesion at baseline showed a large subcutaneous mass originating from the left upper eyelid and reaching the temporal region. It had a gray-rosy color, indistinct margins, soft elastic consistency and was not painful to the touch. A brain MRI at baseline (Figure 4a,c,e) showed pseudo-nodular formations (neurofibromas) in the left fronto-parietal-temporal area, extending to the ipsilateral orbital region. Six months later, the brain MRI (Figure 4b,d,f) showed a clear volume reduction in this plexiform neurofibroma, especially visible in axial TSE T2 weighted slices of the inferior portion (Figure 4e,f). The total volume reduction was around 28%.

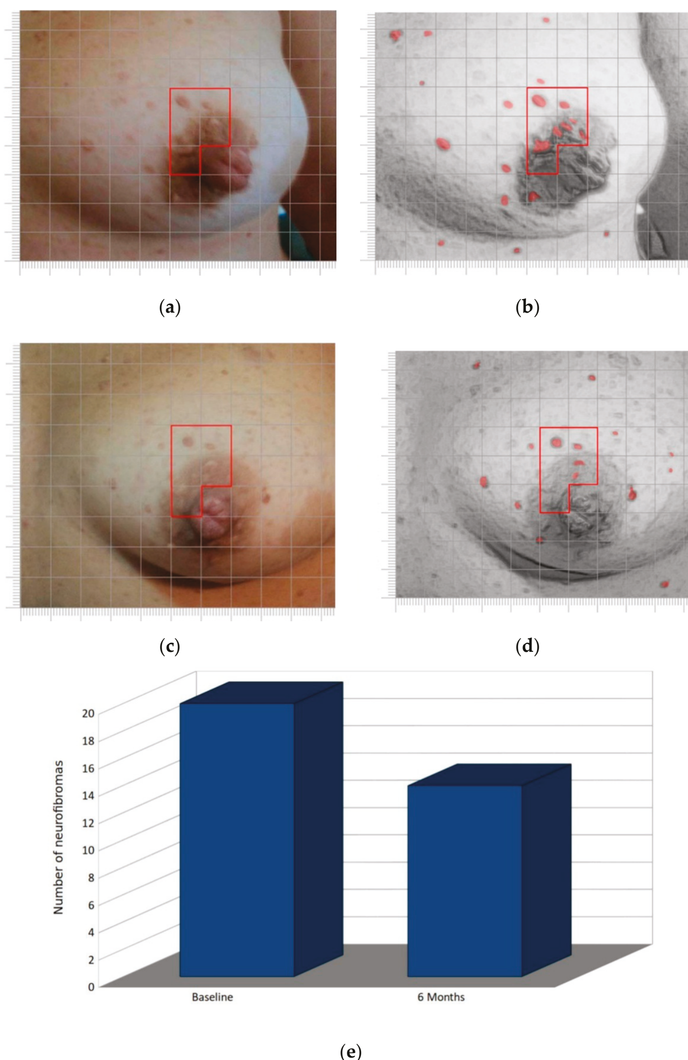


Figure 3. Left breast of case 10: serial clinical assessment. (**a**,**c**). Clinical observation at baseline (**a**) and after six months of MedDietCurcumin (**c**); the red border is provided for reference; (**b**,**d**) Digital reworking of the image in A and C to facilitate manual counting of cutaneous neurofibromas; (**e**) Bar chart representing the number of neurofibromas in the sampled area at baseline and at six-month follow-up.

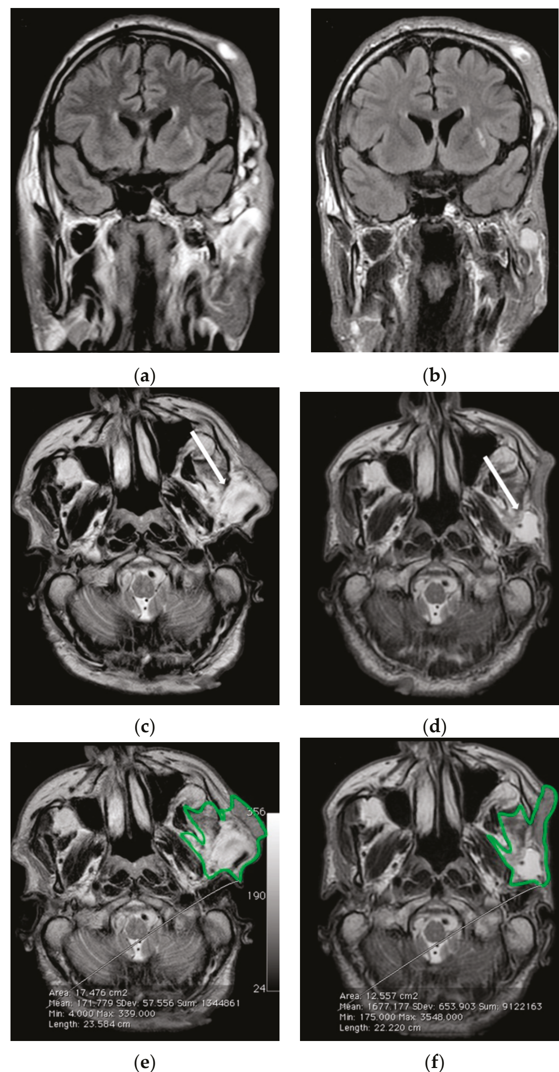


Figure 4. Serial MR imaging assessment of case 11. (**a**) Coronal FLAIR-weighted and (**c**,**e**) Axial TSE T2-weighted images of the left facial plexiform neurofibroma at baseline; (**b**) Coronal FLAIR-weighted and (**d**,**f**) Axial TSE T2-weighted images after six months of MedDietCurcumin. After six months there was a significant reduction especially in the hyperintense parts of the lesion, as indicated by arrows in (**c**,**d**). In (**e**,**f**) a green border contours the area used for volume measurement.

3.2. Nutritional and Bioimpedentiometric Data

From a metabolic point of view, as shown in Table 2, no significant changes in BMI in the WesDiet group (patients 1 and 2) or WesDietCurcumin (6–8) were observed.

Table 2. Bioimpedentiometric and metabolic analysis in the course of follow-up.

Diet	Patient ID	Age (Years)	Sex	Time	% Cell Mass	% Tot Water	% Extracell Water	% Intracell Water	% Fat	% FFM [1]	BMR [1]	BMI [1]	PA [1]	NA/K
WesDiet	1 A.S.	*38*	*M*	*Baseline*	52.3	54	49.7	50.3	26.7	73.3	1235	25	5.7	1.2
				6 months	51.8	53	53.2	46.8	27.2	72.8	1198	25.4	5.5	1.3
	2 A.F.	*22*	*M*	*Baseline*	49.7	46.8	56.2	43.8	32.7	63.7	1065	28	6	1.2
				6 months	48.2	44.6	55.8	44.2	35.6	64.4	998.6	28.5	5.8	1.2
MedDiet	3 E.M.	*27*	*M*	*Baseline*	49.7	54	48.3	51.7	24.6	75.4	1324	26	5.5	1.2
				6 months	51.2	59.6	45.7	54.3	21.2	78.8	1435	23	6.1	0.9
	4 B.E.	*18*	*M*	*Baseline*	46.8	42.8	52	48	43.5	56.5	1129	36	4.8	1.3
				6 months	51.4	45.7	46	54	37.8	62.2	1326	30	5.8	1
	5 D.M.M.	*22*	*M*	*Baseline*	42.7	54.1	53.3	46.7	26.8	73.2	1223	27	5.9	1.1
				6 months	43.4	56.7	46.8	53.2	25	75	1276	26.7	6	1.1
WesDietCurcumin	6 A.M.C.	*41*	*F*	*Baseline*	50.9	58	48	52	21.4	79.6	1345.2	25	5.7	1.1
				6 months	51.6	59	47.2	52.8	20.9	79.1	1365	24.9	5.6	1.2
	7 A.N.	*59*	*M*	*Baseline*	47	48.8	54	46	38.6	61.4	1237	32	4.9	1.2
				6 months	47.1	47.6	55	45	38	62	1195	31.7	4.8	1.1
	8 A.F.	*44*	*F*	*Baseline*	49.2	48.7	50	50	34.4	65.6	1356	30	5.3	0.9
				6 months	50	48.2	48.5	51.5	33.6	66.4	1376.2	29.7	5.4	1
MedDietCurcumin	9 P.C.	*54*	*F*	*Baseline*	41.2	55.2	50.2	49.8	24.8	75.2	956.4	23	4.5	1.3
				6 months	51.5	59.3	48	52	19	81	1250.6	22	5.5	1.2
	10 F.L.	*27*	*F*	*Baseline*	49.1	45.4	49	51	38	62	1343.3	34	5.3	1
				6 months	57.1	48.3	45	55	34.1	65.9	1519.4	31	6.1	0.9
	11 R.S.	*50*	*M*	*Baseline*	50.2	59	47.2	52.8	19.4	80.6	1276.7	24	5.7	1.1
				6 months	52.2	64.4	47.6	52.4	12	88	1407.6	23	5.6	1.3

[1] FFM: Fat-Free Mass; BMR: Basal Metabolic Rate; PA: Phase Angle; BMI: Body Mass Index.

All patients following a Mediterranean diet with curcumin supplementation (9, 10 and 11) or without curcumin (3, 4 and 5) presented a general improvement in their metabolic status, with a BMI reduction and an increase in body hydration, due to the greater intake of water; in all of these patients, the percentage of fat-free mass (FFM), and basal metabolism (BMR) increased. Phase angle (PA) improved in the Mediterranean and MedDietCurcumin patients, with the exception of patient 11, for whom it remained stable. Na/K exchange remained essentially unchanged in all of these patients.

No patient reported any side effects of curcumin consumption

3.3. Plasma Curcuminoid Level

The curcumin supplemented dietary regimen was established on the basis of previous clinical studies for inflammatory conditions, where active dosages of around 1–2 g/day of curcuminoid were used [19,20]. For this study, subjects consumed, in association with Western or traditional Mediterranean-style diet, 1200 mg total curcuminoids. Once absorbed, curcumin is subjected to conjugations like sulfation and glucuronidation at various tissue sites. For this reason, all plasma samples were treated with Helix pomatia glucuronidase/sulfatase before HPLC analysis. Our data (Figure 5) demonstrated that the plasma level of curcumin increased linearly over sixth months in patients who consumed the MedDietCurcumin, reaching a final plasma concentration ranging from 49.2 ± 1.0 ng/mL to 79.0 ± 3.6 ng/mL. Conversely, patients treated with a Western diet supplemented with curcumin showed very low plasma concentrations of curcumin even after sixth months of diet. Taken together, our results demonstrate that a traditional Mediterranean-style diet improves curcuminoid bioavailability.

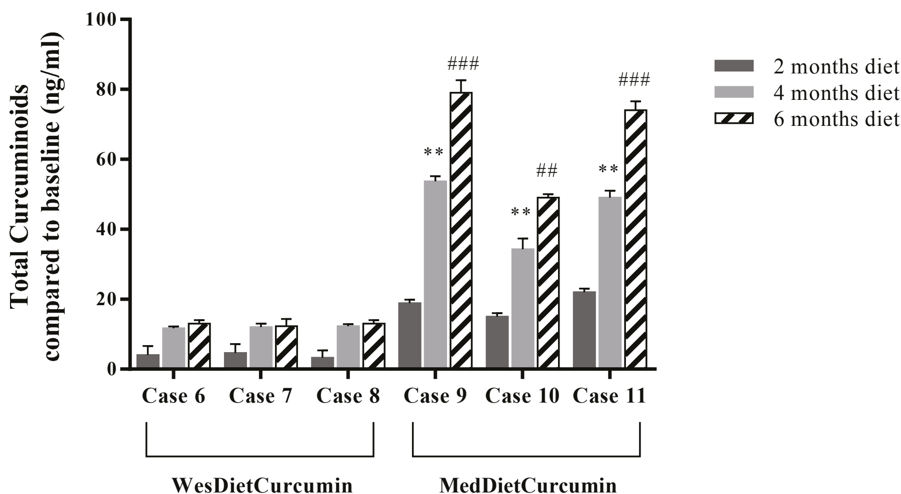


Figure 5. Plasma levels of curcuminoids. HPLC analysis of enzymatically hydrolyzed plasma samples. For each sample, three different experiments were conducted and the results expressed as the mean of the values obtained (mean ± SD). Statistically significant variations: ** $p < 0.01$ 4 months diet versus 2 months diet; ## $p < 0.01$ 6 months diet versus 2 months diet; ### $p < 0.001$ 6 months diet versus 2 months diet.

4. Discussion

The association between NF1 and malignant tumors (gliomas, malignant peripheral nerve sheath tumors (MPNST), leukemia and rhabdomyosarcoma) has been largely described [24]. The mutation is highly penetrant: by age 20, almost 100% of the mutation carriers will manifest the disease in

some form [25]. NF1 is notable for its high phenotypic variability, both within and between families, which means that family members carrying the exact same mutation may present with vastly different clinical pictures [26]. Multiple factors have been put forward as mechanisms underlying this variability, including modifying genes [27], allelic heterogeneity, mutation in the second allele, somatic mosaicism, epigenetic events and exposure to environmental agents [28,29].

Here we report the beneficial effects of a traditional Mediterranean Diet (MedDiet) enriched with curcumin (MedDietCurcumin) on the number and size of neurofibromas in all NF1 patients consuming this diet (patients 9–11). Indeed, besides the improvement in the general metabolic status, and particularly of the lipid profile, we observed a marked reduction in the number (ranging from 30 to 51%) and volume of neurofibromas after six months of MedDietCurcumin. Notably, the large cranial plexiform neurofibroma in patient 11 exhibited a marked volume reduction (28.2%), as shown by a conventional imaging method. In contrast, we observed no significant effect on the pattern of neurofibromas in the patients who did not follow MedDietCurcumin.

Epidemiological evidence and many case-control studies suggest that a Mediterranean diet plays a pivotal role in lowering the risk of several chronic diseases, including cardiovascular disease, neurodegenerative disease, diabetes, and cancer. A high intake of olive oil is considered a hallmark of the traditional Mediterranean diet. In this study, we used extra-virgin olive oil (EVO—the juice of the olive obtained solely by pressing and consumed without any further refining process) having >2000 mg/kg total phenol concentration. It has been reported that EVO consumption increases the monounsaturated fatty acid content in phospholipids and cholesterol esters by modifying the fatty acid composition of the plasma membrane, which influences the association of G proteins and PKCa with the lipid bilayer in elderly persons with type 2 diabetes [30]. The authors demonstrated that after consuming EVO for 4 weeks, the patients showed a significant increase in the total amount of monounsaturated fatty acid, mostly due to a rise in the proportion of oleic acid and a decrease in saturated fatty acids, which influences the membrane fluidity [31]. In addition, the EVO used contained a high level of naturally occurring phenolic compounds, the key feature of prevention of a number of diseases and pathological conditions (i.e., cancer and several aging-associated degenerative diseases) [32,33]. Increasing studies highlight the anti-proliferative and pro-apoptotic effects of the two major EVO components, oleuropein and hydroxytyrosol, on cancer cells and show that these effects stem from different mechanisms depending on the cell type. For example, these polyphenols are able to reduce angiogenesis via downregulation of cyclooxygenase-2 (COX-2) expression, prostanoid production and matrix metallopeptidase 9 (MMP-9) protein release, together with a reduction in intracellular ROS levels and NFκB activation [34–37]. Moreover, polyphenols stimulate apoptosis by activating pro-apoptotic Bcl-2 family members and PI3K/AKT signaling in pancreatic cancer and hepatoma cells, and increasing the c-Jun-N-terminal kinase (cJNK), p53, p21, Bax and cytochrome c cytoplasmic concentration in HeLa and cervix carcinoma cells [38–40]. Furthermore, in breast cancer cells, p53 or the G protein-coupled estrogen receptor 1/30 (GPER1/GPR30) pathway activation has also been shown, as well as the inhibition of the anti-apoptotic and pro-proliferation protein NFκB and cyclin D1, its main oncogenic target [41]. Moreover, the ability of curcumin to target multiple signaling pathways that are linked to tumorigenesis in NF1 represents a promising avenue for therapeutic intervention [42,43].

In particular, curcumin has been extensively studied for its role in Ras oncogenic signaling pathways, notably by abolishing the RAS-ERK signaling mechanism [44]. Depending on the cell type and stimulus, ERK activity mediates different antiproliferative events, such as apoptosis, autophagy and senescence in vitro and in vivo [45]. Findings in the last decade have demonstrated intriguing effects of curcumin in PI3K/AKT/mTOR signaling [46,47]. For instance, curcumin induces G2-M arrest and autophagy in malignant glioma cells through the inhibition of Akt/mTOR/p70S6K and activation of the extracellular signal-regulated kinase (ERK)1/2 pathways, suggesting that autophagy-mediated cell death might be pathway-specific [48]. In addition, preclinical studies show that curcumin induces apoptosis and G2–M arrest in cancer cells by generating superoxides, increasing

caspase-3, caspase-7, and PARP cleavage, downregulating Akt phosphorylation, and upregulating p53 phosphorylation [49]. Curcumin also promotes selective tumor cell death and inhibits proliferation of a human hepatocellular carcinoma cell line (Huh7 cells), providing unequivocal evidence of intricate cross-talk between autophagy and cell death [50]. Another study demonstrated that curcumin increases the sensitivity of neurofibromin deficient MPNST cells to TRAIL (TNF-related apoptosis-inducing ligand), downregulating anti-apoptotic proteins [51,52]. Curcumin may also exert an anti-proliferative effect by decreasing the enzymatic activity of the epidermal growth factor receptor (EGF-R). In fact, NF1 Schwann cells exhibit an aberrant EGF-R expression, which has been linked to increased cellular proliferation and malignancy [53].

Unfortunately, while curcumin is one of the most used nutraceuticals, its utility as a therapeutic agent is limited by its poor water solubility, short biological half-life, and low bioavailability after oral administration in certain tissues. Therefore, various approaches including the use of adjuvants, liposomes, nanoparticles, phospholipid complexes and reformulation with various oils have been tried [54]. Interestingly, our six-month observation results show that only the patients who followed the MedDietCurcumin exhibited a plasmatic increase in curcumin concentration, indicating an improvement of curcuminoid bioavailability. We can speculate that the simultaneous presence of a high dietary concentration of EVO polyphenols and/or fatty acids contributes to this enhancement, and, consequently, to the positive effect on reducing NF1 symptoms. However, this study has some limitations, such as the small number of participants and the short-term exposure to MedDietCurcumin, but we can hypothesize that the regular consumption of polyphenol-rich olive oil and curcumin can maintain the effects observed in this study.

5. Conclusions

NF1 presents a unique situation in which the existence of widespread neurofibromas, and their tendency to recur, calls for more effective and sustainable medical intervention than the simple surgical removal of individual lesions. The results presented in this study are of particular interest as they are the first clinical demonstration of the therapeutic activity on NF1 of curcumin in combination with a dietary approach rich in polyphenols.

As shown by our data, the plasma level of curcumin increased in patients following MedDietCurcumin; this suggests that curcuminoid bioavailability is positively influenced by polyphenol-rich foods in the Mediterranean diet. However, this synergism between curcumin and polyphenols needs to be confirmed on a larger cohort of NF1 patients.

Further studies are necessary to elucidate the molecular mechanisms by which MedDietCurcumin is able to reduce both the number and volume of neurofibromas. In addition, clinical trials involving new nanoformulations of curcumin conjugated with long-term observations may provide further data on the potential therapeutic role of curcumin in NF1 as well as in other inherited or sporadic cancer syndromes.

Acknowledgments: We express our most sincere thanks to the patients for their cooperation in this study. Furthermore, we are grateful to Nicola Mallardo for surgical procedures, Enrica Barra for tissue analysis and Elizabeth McGarry for her critical reading of the manuscript, Giuseppina Franzese and Giovanni Panella for their technical assistance.

Author Contributions: The manuscript was written by Carla Schettino, Teresa Esposito and Paola Polverino. Mariarosa Anna Beatrice Melone, Teresa Esposito, and Gianfranco Peluso conceived and designed the work. The collection of data was assisted by Teresa Esposito, Salvatore Allocca, Alessandra D'Amico, Giuseppe Sorrentino, Giacomo Lus, Laura Adelfi, Guglielmo Capaldo and Carla Schettino. Anna Di Salle performed the plasma analysis of curcuminoids. Mariarosa Anna Beatrice Melone, Bruno Varriale, Giuseppe Di Iorio, Simone Sampaolo and Gianfranco Peluso reviewed the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Conte, R.; De Luca, I.; De Luise, A.; Petillo, O.; Calarco, A.; Peluso, G. New Therapeutic Potentials of Nanosized Phytomedicine. *J. Nanosci. Nanotechnol.* **2016**, *16*, 8176–8187. [CrossRef]
2. Momtazi, A.A.; Derosa, G.; Maffioli, P.; Banach, M.; Sahebkar, A. Role of microRNAs in the Therapeutic Effects of Curcumin in Non-Cancer Diseases. *Mol. Diagn. Ther.* **2016**, *20*, 335–345. [CrossRef] [PubMed]
3. Sahebkar, A.; Cicero, A.F.; Simental-Mendia, L.E.; Aggarwal, B.B.; Gupta, S.C. Curcumin downregulates human tumor necrosis factor-alpha levels: A systematic review and meta-analysis ofrandomized controlled trials. *Pharmacol. Res.* **2016**, *107*, 234–242. [CrossRef] [PubMed]
4. Sahebkar, A. Autophagic activation: A key piece of the puzzle for the curcumin-associated cognitive enhancement? *J. Psychopharmacol.* **2016**, *30*, 93–94. [CrossRef] [PubMed]
5. Sahebkar, A. Curcuminoids for the management of hypertriglyceridaemia. *Nat. Rev. Cardiol.* **2014**, *11*, 123. [CrossRef] [PubMed]
6. Derosa, G.; Maffioli, P.; Simental-Mendia, L.E.; Bo, S.; Sahebkar, A. Effect of curcumin on circulating interleukin-6 concentrations: A systematic review and meta-analysis of randomized controlled trials. *Pharmacol. Res.* **2016**, *111*, 394–404. [CrossRef] [PubMed]
7. Chuang, S.E.; Kuo, M.L.; Hsu, C.H.; Chen, C.R.; Lin, J.K.; Lai, G.M.; Hsieh, C.Y.; Cheng, A.L. Curcumin-containing diet inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. *Carcinogenesis* **2000**, *21*, 331–335. [CrossRef] [PubMed]
8. Casaburi, I.; Puoci, F.; Chimento, A.; Sirianni, R.; Ruggiero, C.; Avena, P.; Pezzi, V. Potential of olive oil phenols as chemopreventive and therapeutic agents against cancer: A review of in vitro studies. *Mol. Nutr. Food Res.* **2013**, *57*, 71–83. [CrossRef] [PubMed]
9. Bayet-Robert, M.; Morvan, D. Metabolomics reveals metabolic targets and biphasic responses in breast cancer cells treated by curcumin alone and in association with docetaxel. *PLoS ONE* **2013**, *8*, e57971. [CrossRef] [PubMed]
10. Huang, Y.T.; Lin, Y.W.; Chiu, H.M.; Chiang, B.H. Curcumin Induces Apoptosis of Colorectal Cancer Stem Cells by Coupling with CD44 Marker. *J. Agric. Food Chem.* **2016**, *64*, 2247–2253. [CrossRef] [PubMed]
11. Buhrmann, C.; Kraehe, P.; Lueders, C.; Shayan, P.; Goel, A.; Shakibaei, M. Curcumin suppresses crosstalk between colon cancer stem cells and stromal fibroblasts in the tumor microenvironment: Potential role of EMT. *PLoS ONE* **2014**, *9*, e107514. [CrossRef] [PubMed]
12. Starok, M.; Preira, P.; Vayssade, M.; Haupt, K.; Salome, L.; Rossi, C. EGFR Inhibition by Curcumin in Cancer Cells: A Dual Mode of Action. *Biomacromolecules* **2015**, *16*, 1634–1642. [CrossRef] [PubMed]
13. Jett, K.; Friedman, J.M. Clinical and genetic aspects of neurofibromatosis 1. *Genet. Med.* **2010**, *12*, 1–11. [CrossRef] [PubMed]
14. Lin, A.L.; Gutmann, D.H. Advances in the treatment of neurofibromatosis-associated tumours. *Nat. Rev. Clin. Oncol.* **2013**, *10*, 616–624. [CrossRef] [PubMed]
15. De Souza, M.L.; Jansen, A.K.; Martins, A.S.; Rodrigues, L.O.; De Rezende, N.A. Nutrient intake in neurofibromatosis type 1: A cross-sectional study. *Nutrition* **2015**, *31*, 858–862. [CrossRef] [PubMed]
16. Carotenuto, M.; Esposito, M. Nutraceuticals safety and efficacy in migraine without aura in a population of children affected by neurofibromatosis type I. *Neurolog. Sci.* **2013**, *34*, 1905–1909. [CrossRef] [PubMed]
17. Esposito, T.; Piluso, G.; Saracino, D.; Uccello, R.; Schettino, C.; Dato, C.; Capaldo, G.; Giugliano, T.; Varriale, B.; Paolisso, G.; et al. A novel diagnostic method to detect truncated neurofibromin in neurofibromatosis 1. *J. Neurochem.* **2015**, *135*, 1123–1128. [CrossRef] [PubMed]
18. Conforti, R.; Cirillo, M.; Marrone, V.; Galasso, R.; Capaldo, G.; Giugliano, T.; Scuotto, A.; Piluso, G.; Melone, M.A. Giant thrombosed intracavernous carotid artery aneurysm presenting as Tolosa-Hunt syndrome in a patient harboring a new pathogenic neurofibromatosis type 1 mutation: A case report and review of the literature. *Neuropsychiatr. Dis. Treat.* **2014**, *10*, 135–140. [CrossRef] [PubMed]
19. Cuomo, J.; Appendino, G.; Dern, A.S.; Schneider, E.; Mckinnon, T.P.; Brown, M.J.; Togni, S.; Dixon, B.M. Comparative absorption of a standardized curcuminoid mixture and its lecithin formulation. *J. Nat. Prod.* **2011**, *74*, 664–669. [CrossRef] [PubMed]
20. Burgos-Moron, E.; Calderon-Montano, J.M.; Salvador, J.; Robles, A.; Lopez-Lazaro, M. The dark side of curcumin. *Int. J. Cancer* **2010**, *126*, 1771–1775. [CrossRef] [PubMed]

21. Heath, D.D.; Pruitt, M.A.; Brenner, D.E.; Rock, C.L. Curcumin in plasma and urine: Quantitation by high-performance liquid chromatography. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2003**, *783*, 287–295. [CrossRef]
22. Lama, G.; Esposito Salsano, M.; Grassia, C.; Calabrese, E.; Grassia, M.G.; Bismuto, R.; Melone, M.A.; Russo, S.; Scuotto, A. Neurofibromatosis type 1 and optic pathway glioma. A long-term follow-up. *Minerva Pediatr.* **2007**, *59*, 13–21. [PubMed]
23. D'arco, F.; D'amico, A.; Caranci, F.; Di Paolo, N.; Melis, D.; Brunetti, A. Cerebrovascular stenosis in neurofibromatosis type 1 and utility of magnetic resonance angiography: Our experience and literature review. *Radiol. Med.* **2014**, *119*, 415–421. [CrossRef] [PubMed]
24. Perry, A.; Roth, K.A.; Banerjee, R.; Fuller, C.E.; Gutmann, D.H. NF1 deletions in S-100 protein-positive and negative cells of sporadic and neurofibromatosis 1 (NF1)-associated plexiform neurofibromas and malignant peripheral nerve sheath tumors. *Am. J. Pathol.* **2001**, *159*, 57–61. [CrossRef]
25. Korf, B.R. The phakomatoses. *Clin. Dermatol.* **2005**, *23*, 78–84. [CrossRef] [PubMed]
26. Wallace, M.R. Neurofibromatosis: Phenotype, Natural History, and Pathogenesis. *Am. J. Hum. Genet.* **2000**, *67*, 264.
27. Easton, D.F.; Ponder, M.A.; Huson, S.M.; Ponder, B.A. An analysis of variation in expression of neurofibromatosis (NF) type 1 (NF1): Evidence for modifying genes. *Am. J. Hum. Genet.* **1993**, *53*, 305–313. [PubMed]
28. Reuter, S.; Gupta, S.C.; Park, B.; Goel, A.; Aggarwal, B.B. Epigenetic changes induced by curcumin and other natural compounds. *Genes Nutr.* **2011**, *6*, 93–108. [CrossRef] [PubMed]
29. Laycock-Van Spyk, S.; Thomas, N.; Cooper, D.N.; Upadhyaya, M. Neurofibromatosis type 1-associated tumours: Their somatic mutational spectrum and pathogenesis. *Hum. Genom.* **2011**, *5*, 623–690. [CrossRef]
30. Perona, J.S.; Vogler, O.; Sanchez-Dominguez, J.M.; Montero, E.; Escriba, P.V.; Ruiz-Gutierrez, V. Consumption of virgin olive oil influences membrane lipid composition and regulates intracellular signaling in elderly adults with type 2 diabetes mellitus. *J. Gerontol. A Biol. Sci. Med. Sci.* **2007**, *62*, 256–263. [CrossRef] [PubMed]
31. Muriana, F.J.; Vazquez, C.M.; Ruiz-Gutierrez, V. Fatty acid composition and properties of the liver microsomal membrane of rats fed diets enriched with cholesterol. *J. Biochem.* **1992**, *112*, 562–567. [CrossRef] [PubMed]
32. Rahmani, A.H.; Albutti, A.S.; Aly, S.M. Therapeutics role of olive fruits/oil in the prevention of diseases via modulation of anti-oxidant, anti-tumour and genetic activity. *Int. J. Clin. Exp. Med.* **2014**, *7*, 799–808. [PubMed]
33. Goldsmith, C.D.; Vuong, Q.V.; Sadeqzadeh, E.; Stathopoulos, C.E.; Roach, P.D.; Scarlett, C.J. Phytochemical properties and anti-proliferative activity of Olea europaea L. leaf extracts against pancreatic cancer cells. *Molecules* **2015**, *20*, 12992–13004. [CrossRef] [PubMed]
34. Marchetti, C.; Clericuzio, M.; Borghesi, B.; Cornara, L.; Ribulla, S.; Gosetti, F.; Marengo, E.; Burlando, B. Oleuropein-Enriched Olive Leaf Extract Affects Calcium Dynamics and Impairs Viability of Malignant Mesothelioma Cells. *Evid. Based Complem. Altern. Med.* **2015**, *2015*, 908493. [CrossRef] [PubMed]
35. Curic, S.; Wu, Y.; Shan, B.; Schaaf, C.; Utpadel, D.; Lange, M.; Kuhlen, D.; Perone, M.J.; Arzt, E.; Stalla, G.K.; et al. Curcumin acts anti-proliferative and pro-apoptotic in human meningiomas. *J. Neurooncol.* **2013**, *113*, 385–396. [CrossRef] [PubMed]
36. Scoditti, E.; Calabriso, N.; Massaro, M.; Pellegrino, M.; Storelli, C.; Martines, G.; De Caterina, R.; Carluccio, M.A. Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: A potentially protective mechanism in atherosclerotic vascular disease and cancer. *Arch. Biochem. Biophys.* **2012**, *527*, 81–89. [CrossRef] [PubMed]
37. Chimento, A.; Casaburi, I.; Rosano, C.; Avena, P.; De Luca, A.; Campana, C.; Martire, E.; Santolla, M.F.; Maggiolini, M.; Pezzi, V.; et al. Oleuropein and hydroxytyrosol activate GPER/ GPR30-dependent pathways leading to apoptosis of ER-negative SKBR3 breast cancer cells. *Mol. Nutr. Food Res.* **2014**, *58*, 478–489. [CrossRef] [PubMed]
38. Aoki, H.; Takada, Y.; Kondo, S.; Sawaya, R.; Aggarwal, B.B.; Kondo, Y. Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: Role of Akt and extracellular signal-regulated kinase signaling pathways. *Mol. Pharmacol.* **2007**, *72*, 29–39. [CrossRef] [PubMed]

39. Yan, C.M.; Chai, E.Q.; Cai, H.Y.; Miao, G.Y.; Ma, W. Oleuropein induces apoptosis via activation of caspases and suppression of phosphatidylinositol 3-kinase/protein kinase B pathway in HepG2 human hepatoma cell line. *Mol. Med. Rep.* **2015**, *11*, 4617–4624. [CrossRef] [PubMed]
40. Yao, J.; Wu, J.; Yang, X.; Yang, J.; Zhang, Y.; Du, L. Oleuropein induced apoptosis in HeLa cells via a mitochondrial apoptotic cascade associated with activation of the c-Jun NH2-terminal kinase. *J. Pharmacol. Sci.* **2014**, *125*, 300–311. [CrossRef] [PubMed]
41. Hassan, Z.K.; Elamin, M.H.; Omer, S.A.; Daghestani, M.H.; Al-Olayan, E.S.; Elobeid, M.A.; Virk, P. Oleuropein induces apoptosis via the p53 pathway in breast cancer cells. *Asian Pac. J. Cancer Prev.* **2014**, *14*, 6739–6742. [CrossRef] [PubMed]
42. Monroe, C.L.; Dahiya, S.; Gutmann, D.H. Dissecting Clinical Heterogeneity in Neurofibromatosis Type 1. *Annu. Rev. Pathol.* **2017**, *12*, 53–74. [CrossRef] [PubMed]
43. Kasi, P.D.; Tamilselvam, R.; Skalicka-Wozniak, K.; Nabavi, S.F.; Daglia, M.; Bishayee, A.; Pazoki-Toroudi, H.; Nabavi, S.M. Molecular targets of curcumin for cancer therapy: An updated review. *Tumor Biol.* **2016**, *37*, 13017–13028. [CrossRef] [PubMed]
44. Cao, A.L.; Tang, Q.F.; Zhou, W.C.; Qiu, Y.Y.; Hu, S.J.; Yin, P.H. Ras/ERK signaling pathway is involved in curcumin-induced cell cycle arrest and apoptosis in human gastric carcinoma AGS cells. *J. Asian Nat. Prod. Res.* **2015**, *17*, 56–63. [CrossRef] [PubMed]
45. Cagnol, S.; Chambard, J.C. ERK and cell death: Mechanisms of ERK-induced cell death—Apoptosis, autophagy and senescence. *FEBS J.* **2010**, *277*, 2–21. [CrossRef] [PubMed]
46. Polivka, J., Jr.; Janku, F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. *Pharmacol. Ther.* **2014**, *142*, 164–175. [CrossRef] [PubMed]
47. Yu, S.; Shen, G.; Khor, T.O.; Kim, J.H.; Kong, A.N. Curcumin inhibits Akt/mammalian target of rapamycin signaling through protein phosphatase-dependent mechanism. *Mol. Cancer Ther.* **2008**, *7*, 2609–2620. [CrossRef] [PubMed]
48. Hasima, N.; Ozpolat, B. Regulation of autophagy by polyphenolic compounds as a potential therapeutic strategy for cancer. *Cell Death Dis.* **2014**, *5*, e1509. [CrossRef] [PubMed]
49. Weir, N.M.; Selvendiran, K.; Kutala, V.K.; Tong, L.; Vishwanath, S.; Rajaram, M.; Tridandapani, S.; Anant, S.; Kuppusamy, P. Curcumin induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by modulating Akt and p38 MAPK. *Cancer Biol. Ther.* **2007**, *6*, 178–184. [CrossRef] [PubMed]
50. Rainey, N.; Motte, L.; Aggarwal, B.B.; Petit, P.X. Curcumin hormesis mediates a cross-talk between autophagy and cell death. *Cell Death Dis.* **2015**, *6*, e2003. [CrossRef] [PubMed]
51. Dai, X.; Zhang, J.; Arfuso, F.; Chinnathambi, A.; Zayed, M.E.; Alharbi, S.A.; Kumar, A.P.; Ahn, K.S.; Sethi, G. Targeting TNF-related apoptosis-inducing ligand (TRAIL) receptor by natural products as a potential therapeutic approach for cancer therapy. *Exp. Biol. Med. (Maywood)* **2015**, *240*, 760–773. [CrossRef] [PubMed]
52. Reuss, D.E.; Mucha, J.; Hagenlocher, C.; Ehemann, V.; Kluwe, L.; Mautner, V.; Von Deimling, A. Sensitivity of malignant peripheral nerve sheath tumor cells to TRAIL is augmented by loss of NF1 through modulation of MYC/MAD and is potentiated by curcumin through induction of ROS. *PLoS ONE* **2013**, *8*, e57152. [CrossRef] [PubMed]
53. Ling, B.C.; Wu, J.; Miller, S.J.; Monk, K.R.; Shamekh, R.; Rizvi, T.A.; Decourten-Myers, G.; Vogel, K.S.; Declue, J.E.; Ratner, N. Role for the epidermal growth factor receptor in neurofibromatosis-related peripheral nerve tumorigenesis. *Cancer Cell* **2005**, *7*, 65–75. [CrossRef] [PubMed]
54. Conte, R.; Calarco, A.; Napoletano, A.; Valentino, A.; Margarucci, S.; Di Cristo, F.; Di Salle, A.; Peluso, G. Polyphenols Nanoencapsulation for Therapeutic Applications. *J. Biomol. Res. Ther.* **2016**, *5*. [CrossRef]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Unfolding Novel Mechanisms of Polyphenol Flavonoids for Better Glycaemic Control: Targeting Pancreatic Islet Amyloid Polypeptide (IAPP)

Ivana R. Sequeira [1,2] and Sally D. Poppitt [1,2,3,*]

[1] Human Nutrition Unit, School of Biological Sciences, University of Auckland, Auckland 1010, New Zealand; i.sequeira@auckland.ac.nz
[2] High-Value Nutrition National Science Challenge, Auckland 1142, New Zealand
[3] Department of Medicine, University of Auckland, Auckland 1142, New Zealand
[*] Correspondence: s.poppitt@auckland.ac.nz; Tel.: +64-(09)-630-5160

Received: 14 June 2017; Accepted: 18 July 2017; Published: 21 July 2017

Abstract: Type 2 diabetes (T2D) is characterised by hyperglycaemia resulting from defective insulin secretion, insulin resistance, or both. The impact of over-nutrition and reduced physical activity, evidenced by the exponential rise in obesity and the prevalence of T2D, strongly supports the implementation of lifestyle modification programs. Accordingly, an increased consumption of fruits and plant-derived foods has been advocated, as their intake is inversely correlated with T2D prevalence; this has been attributed, in part, to their contained polyphenolic compounds. Over the last decade, a body of work has focussed on establishing the mechanisms by which polyphenolic compounds exert beneficial effects to limit carbohydrate digestion, enhance insulin-mediated glucose uptake, down-regulate hepatic gluconeogenesis and decrease oxidative stress; the latter anti-oxidative property being the most documented. Novel effects on the inhibition of glucocorticoid action and the suppression of amylin misfolding and aggregation have been identified more recently. Amyloid fibrils form from spontaneously misfolded amylin, depositing in islet cells to elicit apoptosis, beta cell degeneration and decrease insulin secretion, with amyloidosis affecting up to 80% of pancreatic islet cells in T2D. Therefore, intervening with polyphenolic compounds offers a novel approach to suppressing risk or progression to T2D. This review gives an update on the emerging mechanisms related to dietary polyphenol intake for the maintenance of glycaemic control and the prevention of T2D.

Keywords: type 2 diabetes; insulin resistance; β-cell dysfunction; polyphenols; flavonoids; protein misfolding disease (PMD); rutin; quercetin-*O*-rutinoside; antioxidant; islet amyloid polypeptide (IAPP); amyloidogenesis

1. Introduction

Worldwide, the number of people diagnosed with diabetes mellitus (DM) has more than doubled over the past three decades [1], with an estimated 285 million individuals in 2010 [2], of which 90% had type 2 diabetes (T2D) [3]. The main pathophysiologic drivers of T2D are insulin resistance (IR) and pancreatic β-cell dysfunction. IR occurs when the body becomes less sensitive or becomes resistant to the action of insulin, manifesting as inadequate insulin-mediated suppression of hepatic gluconeogenesis and inadequate glucose disposal from circulation [4]. Pancreatic β-cells, in turn, are the insulin and amylin-secreting cells of the pancreas, which may alter in both structure and function during a disease state. Each presents at distinct times during the course of disease progression, with IR proposed to be the primary driver and β-cell dysfunction a later manifestation [5]. The etiology of these factors is distinct. IR may result from a defect in insulin signalling, a defect in glucose transporters

or lipotoxicity. Conversely, β-cell dysfunction is postulated to be caused by amyloid deposits in the pancreatic islet's cells, oxidative stress and increased fatty acids within the pancreas, or limited incretin action [6]. Recent studies provide data to suggest that the accumulation of islet amyloid polypeptide (IAPP) or amylin, which is co-secreted with insulin in the pancreatic β-cells, worsens pancreatic function, fast-tracking progression to T2D [7]. Emerging evidence demonstrates that it is the gradual accumulation of the IAPP fibrils, rather than the amyloid deposit itself, that is cytotoxic [8], resulting in increased oxidative stress and membrane permeability [9,10]; both features critical to the pathogenesis of T2D [11]. Accordingly, the role of polyphenol flavonoids is receiving particular interest, given that they have been shown to interfere with the amyloid assembly pathway to inhibit the formation of amylin aggregates, associated cytotoxicity and pancreatic β-cell apoptosis [12,13]. The favourable molecular structure of flavonoids enables them to chemically bind to and prevent assembly of the IAPP fibrils and is now emerging as a possible therapeutic strategy for preventing and delaying progression to T2D [14]. IR most often precedes the onset of T2D and is compensated for by the increased secretion of insulin from islet β-cells to maintain normal circulating glucose levels. This was established in early studies by Reaven and colleagues [15–17] which demonstrated that approximately 25% of nondiabetic individuals exhibit IR within the range of that observed in T2D patients. A deterioration into an inability to regulate blood glucose, both when fasted and following a meal, occurs when either IR increases or β-cell insulin secretory responses decrease, or both.

The alarming increase in the prevalence of T2D, once considered a health issue that plagued Western industrialised nations, has led to significant concern in developing countries [18,19]. On the basis of population growth rates, the aging generation and rates of urbanisation, it has been estimated that two Asian countries—India and China—will shoulder the global T2D burden by the year 2030 [2]. Asian ethnicities appear to be more susceptible to T2D than their Caucasian counterparts, despite commonly being of lower body weight and body mass index (BMI). This may be caused in part by ectopic lipid infiltration into key metabolic organs such as the pancreas and liver [20], the underpinning mechanism for which is as yet not determined but is purported to be due in part to a consequence of lipid 'overspill' from peripheral or central adipose depots during weight gain. One of the key factors attributed to the growth of the diabesity epidemic is a marked change in lifestyle and dietary habits as a result of environmental changes and globalisation [21,22]. Evidence from epidemiological studies indicate that 90% of cases of incident T2D can be attributed to being overweight or obese, over-nutrition, lack of physical activity, smoking, and alcohol consumption [23–25]. Hence, ongoing strategies for the management of T2D emphasise the importance of timely intervention through modifiable risk factors, such as dietary and lifestyle changes [26], which are pertinent to not only delaying progression but also preventing the risk of developing T2D [27]. Robust data from several major diabetes prevention trials [26,28–31] unequivocally show that intensive lifestyle interventions, aimed at weight loss and increased physical activity in high-risk individuals, can prevent or at least delay the progression to overt T2D by 50% [32]. Consequently, considered as effective as intervening with pharmacological agents [33], lifestyle changes are also increasingly promoted as cost-effective [34], affording a maximal benefit with minimal harm [35] to at-risk individuals with poor metabolic health.

The aim of this review is to provide an update on the established mechanisms by which polyphenol flavonoids are known to reduce the risk of T2D and to present a novel mechanism for the inhibition of amylin misfolding and aggregation.

2. Polyphenol Flavonoids Are Essential Non-Nutrient Bioactive Molecules, Having Established Mechanisms in Reducing the Risk of T2D

Several studies highlight the benefit of a diet characterised by not only greater quantities but also a greater variety of fruit and vegetables as an important predictor of glucose tolerance and decreased T2D risk [36–39]. While the precise mechanism by which fruit and vegetables exert their beneficial effects are unknown, the ubiquitous polyphenolic phytochemicals contained within them have been proposed to have favourable effects. Dietary polyphenols constitute approximately 500 compounds

with diverse structure and distribution in foods [40]. As they occur widely in plant-derived foods and beverages, it is estimated that the average intake of polyphenolic compounds in the US population is ~1 g/day [41], between ~0.5–2 g/day in European countries [42], and 0.8–1.1 g/day in the UK [43,44]. Common items in a diet enriched with these non-nutrient bioactive polyphenolic compounds include broccoli, onion, cabbage, grapes, apples, cherries, pears, strawberries, oranges, legumes such as soyabean, cocoa and chocolate [45].

Various epidemiological studies further support the beneficial effects of polyphenol-rich diets in preventing and managing T2D [46]. It was reported in the Nurses' Health Study (N = 1111) (NHS I and II) that markers of flavonones (naringenin and hesperetin) and flavonols (quercetin and isorhamnetin) were significantly associated with a 30–48% lower T2D risk during the follow-up period ($\leq$4.6 years (median)) [47]. Total flavonoid (HR: 0.90; 95% CI 0.77–1.04, p = 0.04) and flavonol (HR: 0.81; 95% CI 0.69–0.95, p = 0.02) intakes were also shown to reduce the risk of T2D in the European prospective investigation into cancer and Nutrition-InterAct (EPIC-InterACT) study [48]. Similarly, higher intakes of anthocyanins and anthocyanin-rich foods were shown to be associated with a significantly lower risk of T2D (pooled HR for 3 cohorts: 0.85 95% CI 0.80–0.91, p < 0.001) in the NHSI (N = 70,359 women), NHSII (N = 89,201 women) and Health Professionals Follow-Up studies (N = 41,334 men) [49]. Whole fruit consumption, such as apples [49–51], pears [49] and blueberries [49,51] which contain polyphenolic compounds are also reported to be inversely associated with T2D. The beneficial effects of total flavonoid intake or any of the flavonoid subclasses are, however, not observed in some studies [50,52]; this may be due to differences in intakes, variability in absorption following dietary intake [53], the structure of the polyphenol itself and the nature of the food source [54]. Notably, these studies [50,52] utilised self-reported semi-quantitative food frequency questionnaires (FFQs)—a tool commonly used to assess dietary intake—that are recognised to be limited in accuracy, as polyphenol intakes may likely be over or under-estimated by this methodology [55].

A large body of evidence links the antioxidant activity of polyphenols [47,49,56–60] as the primary mechanism by which they lower T2D risk. This is in line with results from a recent meta-analysis [61] which confirmed that the consumption of dietary flavonoids was associated with both the maintenance of body weight and a decreased risk of T2D [59]. The beneficial effects of polyphenols in T2D has also been recently reviewed to expand the effects to include improved carbohydrate metabolism via the modulation of metabolic enzymes and nuclear receptors [62], the alteration of gene expression and signalling pathways [63,64], a reduction in the absorption of simple sugars via the inhibition of α-amylase and α-glucosidase, and also an improved uptake of glucose by muscle and adipocytes [46,64]. Furthermore, polyphenol flavonoids also modulate the release of glucose monomers from glycogen deposits by inhibiting glycogen phosphorylase (GP) to prevent hyperglycaemic episodes [65]. Recently, polyphenol-rich olive leaf extracts (OLE) [66–70] have been shown to improve lipid and glycaemic control in T2D, in line with a recent meta-analysis [71] of 36 controlled, randomised trials using polyphenol-extracts, supplements and foods, ranging from 28 mg to 1.5 g for 0.7–12 months, that showed that polyphenol intake lowered Hb_{A1c} levels by 2.29 $\pm$ 0.4 mmol/mol in T2D (N = 1426, baseline Hb_{A1c} = 58 mmol/mol).

3. Unravelling Novel Mechanisms by Which Polyphenol Flavonoids Further Ameliorate T2D Risk

A novel and important target for the use of dietary polyphenol flavonoids in the prevention of T2D is the misfolding of pancreatic amylin and the subsequent deposition of these aggregates in islet β-cells. Indeed, it is their unique aromatic features and their highly conjugated system with multiple hydroxyl groups that make them ideal candidates for targeting amyloid deposits and, additionally, as effective electron and hydrogen atom donors [63] to neutralise free radicals and other reactive oxygen species (ROS) [72]. All polyphenolic compounds share a common phenolic ring structure, with one or more phenolic rings linked to more than one hydroxyl group [73], and are categorised into three main sub-groups: phenolic acids, flavonoids and non-flavonoids. The flavonoids are the largest

class of phenolic compounds and include sub-classes, 6 of which are highlighted according to their nutritional relevance: flavonols, isoflavones, anthocyanidins, flavan-3-ols, flavones and flavanones [53]. Dietary quercetin is the most abundant flavonol and is found in onions, apples, tea, broccoli, and red wine (Table 1) along with kaemferol, isorhamnetin, and myricetin, and is a potent antioxidant [74] (Figure 1).

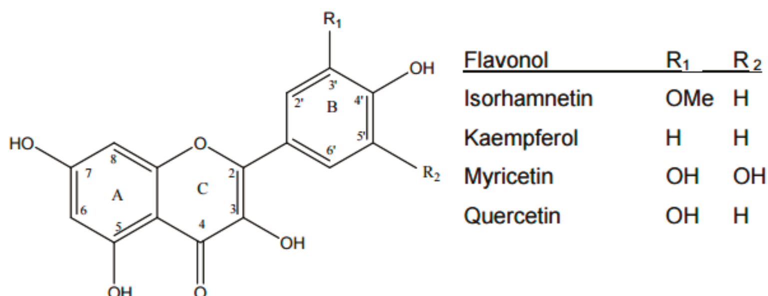


Figure 1. Chemical structure of flavonols: quercetin is the most abundant in the diet and is found in onions, broccoli, apples, tea and red wine.

Table 1. Dietary quercetin and rutin-content in common foods.

Food Source	Quercetin (mg/100 g)	Rutin (mg/100 g)
Apple (with skin) *	3.80	0.22
Broccoli (raw)	2.25	1.6
Buckwheat groats (raw)	3.47	23.0
Grape skin (red)	1.05	149.1
Raspberry (red)	1.10	11.0
Cocoa powder (unsweetened)	10.0	-
Onion (raw)	20.30	0.68
Spinach (raw)	3.97	-
Black tea (brewed) **	2.19	1.62
Green tea (brewed)	2.49	1.46
Fruit tea (pomegranate)	0.00	632
Red wine ***	2.11	0.81

Data obtained from the United States Department of Agriculture and is determined by column or high-performance liquid chromatography, capillary zone electrophoresis, or micellar electrokinetic capillary chromatography [75–80]. * Apples reported as Gala apples. ** Brewed Tea (mg/100 g (100 mL)): tea infusions equivalent to 1 g of dry tea. Infusion values are standardised to 1% infusion (1 g tea leaves/100 mL boiling water) *** Red wine reported as Syrah or Shiraz.

Quercetin commonly occurs conjugated with a sugar moiety at the 5, 7, 3′, 4′, or 5′ positions, which is frequently a rutinoside conjugate (rutin) [81], such that the glycoside combines the flavonol quercetin and the disaccharide rutinose. Rutin is thought to be the main glycosidic form of quercetin in the diet [82] and is found abundantly in buckwheat [83,84] (Table 1). Additionally, quercetin has been shown to have a greater antioxidant activity compared to polyphenolic acids without this structure, due to 3-hydroxy groups and conjugated π bonds [73] (Figure 2); hence the purported beneficial effects of quercetin and quercetin-*O*-rutinoside (rutin) in the improvement of glycaemic indices in recent in vitro [85] and animal models [86–90] studies, as well as in preventing amylin misfolding and aggregation [91–94].

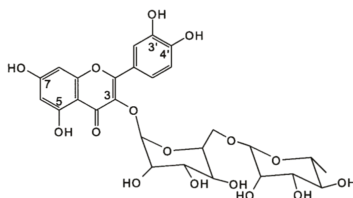


Figure 2. Structure of quercetin-3-*O*-rutinoside (rutin): quercetin commonly occurs conjugated with a sugar moiety at the 5, 7, 3′, 4′ or 5′ position.

3.1. Pancreatic β-Cell Dysfunction Due to Amylin Misfolding and Aggregation

Accumulating evidence suggests that toxic aggregates of islet amyloid polypeptide (IAPP), commonly known as amylin [95], may make a significant contribution to β-cell dysfunction and T2D [8,96–98], it is classified as a protein misfolding disease (PMD). PMDs are a common occurrence in conditions when at least one protein or peptide misfolds, aggregates, and accumulates in tissues where damage occurs. This has also been implicated in the pathogenesis of several neurodegenerative disorders, including Alzheimer's (AD), Parkinson's (PD) and Huntington disease (HD) [99]. Evidence linking protein misfolding and aggregation with disease comes from post-mortem histopathological studies, where a typical feature of each disease is the accumulation of protein deposits; namely, amyloid β (Aβ) in AD, α-synuclein in PD, and poly-Q expanded Huntington in HD [99]. IAPP is a 4 kDa peptide hormone composed of 37-amino acids that is synthesised and co-secreted along with insulin from pancreatic islet β-cells [100]. IAPP has important glucomodulatory effects, as it slows gastric emptying to regulate glucose levels and inhibits the effect of insulin and arginine-stimulated glucagon release by pancreatic α-cells [101]. Moreover, IAPP is involved in appetite regulation via the gut-brain axis and functions as a growth factor in maintaining β-cell mass [102]. The IAPP monomer is shown to have normal biological activity in healthy islet β-cells, wherein oligomers can form and undergo degradation. It is unclear why this process is dysregulated in IR and T2D [103] to the extent that innate physiochemical properties predispose it to aggregate and form fibrils [8], but growing evidence supports this as a key causative driver of T2D. Notably, glucocorticoid (GC) may have a role in pathology [104], much like that observed in AD to form amyloid β [105]. A GC-mediated increase in β-cell IAPP to insulin secretion has been established following dexamethasone treatment in animals [106–108] as well as humans [109]. A major determinant of GC action is the enzymes that convert low active cortisone into active cortisol, especially 11β-hydroxysteroid degydrogenase type 1 (HSD1), mainly expressed in liver and adipose tissue [110], a key mediator of IR [111–113] and pancreatic β-cell dysfunction [114].

The association of IAPP accumulation with T2D was first described as 'islet hyalinosis' by Eugene Opie [115] and reported to be a common occurrence in 90% of T2D individuals [116–118]. Notably, some diabetic patients were reported not to present with islet amyloid [116,119], an important observation which has more recently been explained by the identification of soluble oligomers and aggregates of amylin, which are more cytotoxic than the mature fibrils. Conversely, some normoglycaemic individuals may exhibit these features in line with AD and PD, where disease-free older individuals may also develop protein aggregates [120,121]. Rodent studies show the formation of IAPP aggregates precedes β-cell dysfunction and the clinical signs of disease [122–124], suggesting that hyperglycaemia may not be a prerequisite for islet amyloid formation. This has more recently been verified by careful phenotyping of human amylin (hA) transgenic mice, which showed that hA oligomers did not arise as a result of T2D, but were causative of the dysglycaemia [8]. While evidence from clinical post-mortem studies links IAPP aggregation with a loss of β-cell mass and frank T2D [118,125], it is unlikely that large amyloid deposits are required for β-cell degeneration; instead, evidence points to small soluble oligomers as the likely cytotoxic forms of hA. Studies showing misfolded fibrillogenic hA to be cytotoxic and causative of pancreatic islet β-cell apoptosis have identified several cell-signalling

pathways, including the activation of Fas-associated death receptor signalling [126] confirmed by Fas blocking antibodies, which suppressed hA-evoked apoptosis.

3.2. Mechanisms That Underpin the Formation of Islet Amyloid Aggregates

Amyloidogenesis occurs in three stages, whereby initially the protein misfolds (lag phase) and is rearranged to form the β-sheet (growth phase), which matures to form stable fibrils (saturation phase) or amyloid deposits [14]. Briefly, a model has been proposed for islet amyloid formation [97]. In a healthy state, IAPP is predominantly expressed by pancreatic β-cells as the 89-amino acid, pre-pro-IAPP, which in the endoplasmic reticulum (ER) is cleaved to pro-IAPP and further processed in the Golgi apparatus and secretory granules in a pH-dependent manner. It is noteworthy that cleavage occurs via similar enzymes which process pro-insulin. Processed IAPP is stored with insulin in the secretory granules in a molar ratio of 1-2:50. Insulin and pro-insulin have an inhibitory effect on IAPP aggregation and, together with the low pH within the secretory granules, maintain IAPP in the soluble state. The resultant IAPP is co-secreted along with insulin by the β-cells in response to glucose stimuli.

However, during IR and/or β-cell dysfunction, the expression of IAPP increases to that of insulin, resulting in the misfolding of pro-IAPP in the ER and/or the decreased processing of pro-IAPP in the secretory granule. The resultant misfolded and/or unprocessed pro-IAPP present in the secretory granule is released along with insulin and, extracellularly, further undergoes structural changes to initiate fibril formation. Additionally, misfolded pro-IAPP in the secretory granules may cause the contents within the granules to be targeted to the lysosome to be degraded, as the lysosomal system is responsible for the removal of excess or misfolded peptides, such as IAPP and insulin. Thus, it is also possible that fibril formation could occur intracellularly as a result of the aggregation of pro-IAPP in the lysosome, with the nascent fibrils released into the extracellular space [127]. Once these fibrils are formed either within or outside the β-cells, they provide the 'seed/nucleus' required to facilitate the second stage, or rapid amyloid fibril accumulation [128]. The process of further fibril formation is stabilised by intermolecular hydrogen bonding [129] to form small β-sheet cytotoxic oligomers, which eventually form the amyloid deposits. Alternatively, the protein self-assembly process involves π–π interaction, in which the aromatic residues of IAPP interact with each other via π-stacking to form the amyloid [130] (Figure 3).

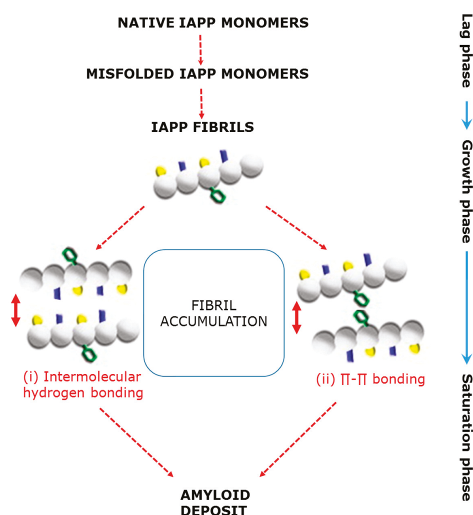


Figure 3. Schematic depiction of the two proposed mechanisms by which IAPP fibrils misfold and aggregate to form amyloidogenic deposits (adapted from [131]).

A recent study of transgenic mice demonstrated that the amount of IAPP produced, along with the degree of oligomerization, differentially affects the amyloid fibril formation and its subsequent cytotoxic effects [8]. This is in line with previous work [132,133] that reported the increased cytotoxicity of oligomeric intermediates rather than the mature fibrils itself. While the exact mechanism of toxicity remains unknown, it has been suggested that the oligomers disrupt the cellular membranes, e.g., mitochondria [127], by forming pore-like structures [9], destabilising the intracellular ionic environment to generate ROS [10] and trigger apoptosis [133–135]. IAPP exposure in rat insulinoma, RINm5F, cells and human islet β-cells [136,137] has been shown to up-regulate pro-apoptotic genes—c-fos, fosB, c-jun, and junB [138]—in a time and concentration-dependent manner, as well as to increase the expression of apoptotic markers p53 and $p21^{WAFI/CIPI}$ [139].

3.3. Targeting Amylin Misfolding and Aggregation with Polyphenol Flavonoids—An Emerging Novel Therapy for T2D

Amyloid deposits have been shown in vivo to be in a dynamic state of turnover and have the potential to regress if fibril formation is inhibited [140]. Hence, preventing or arresting the formation of amyloid-related β-cell failure at an early stage of T2D may preserve endogenous insulin secretion and prevent or delay hyperglycaemia. Two suggested mechanisms involve the inhibition of (i) the precursor pool of IAPP and (ii) the amyloid fibril. The former mechanism is postulated, given that IAPP deficiency in Type 1 diabetics (T1D) does not seem to be associated with severe clinical abnormalities. The evidence supports insulin secretion, and its subsequent effect on the rate of glucose disposal, to be lower in transgenic mice that overproduce hA than that seen in normal mice [141]. Conversely, the reverse is observed in IAPP knockout mice [142], suggesting that, in addition to exerting an anti-amyloidogenic effect, inhibiting the production of IAPP may improve glycaemic control through the inhibition of the potentially diabetogenic metabolic effects of the polypeptide. While demonstrated in vitro, the use of antisense oligonucleotides [143] or the expression of antisense complementary DNA [144] has been proposed for the direct inhibition of IAPP, to increase insulin mRNA and the protein content of cells. Again, IAPP inhibition is also proposed via an indirect mechanism, whereby a reduction in an individual's insulin requirements will in turn reduce the production of IAPP and therefore amyloidosis. This reduction may be initiated by administering insulin therapy early in the course of T2D [145] or by the use of antidiabetic drugs, i.e., metformin.

A more attractive approach clinically is via the dissociation of amyloid fibrils during their formation in order to disrupt the β-pleated sheets and to prevent amyloidosis [146], and has been demonstrated using short synthetic peptides, containing the self-recognition motifs of the protein, engineered to destabilise the abnormal conformation to correct protein misfolding [146]. This has also been shown by binding IAPP monomers with ion ligands [147] to inhibit oligomerisation and effectively reduce amyloid cytotoxicity [148], and is thought to be the mechanism by which tetracycline exerts anti-diabetic activity [91]. A unique feature of tetracycline is that it contains an aromatic ring that facilitates the interaction with lipophilic residues of monomers (π stacking) as polar groups that can form hydrogen bonds with specific residues to strengthen the drug–protein interaction [149,150]. Likewise, polyphenols have been shown to act as small molecule inhibitors to prevent amyloid formation via similar mechanisms. According to the "π stacking" theory, the aromatic rings of polyphenols may competitively interact with aromatic residues in IAPP by sandwiching between two aromatic residues to prevent π–π interaction and block the self-assembly process [92] (Figure 4). Alternatively, the phenolic hydroxyl group of polyphenols may inhibit amyloid fibril formation by binding to the hydrophobic residues in IAPP to modulate oligomerisation [151] (Figure 4). This is similar to the mechanism by which quercetin and epigallocatechin gallate (EGCG) has also been shown to inhibit 11β-HSD1, by binding to the active site by hydrogen bond interaction [152,153].

It is important to consider that the mechanism by which polyphenols inhibit amyloid formation differs depending on which part of the assembly pathway it is involved in [154]. Accordingly, polyphenols have been shown to interact with different forms; i.e., either the monomeric, oligomeric

or fibrillar forms. Some polyphenols have been shown to exert their inhibitory effects on the oligomers, while others inhibit the formation of fibrils and some others do both.

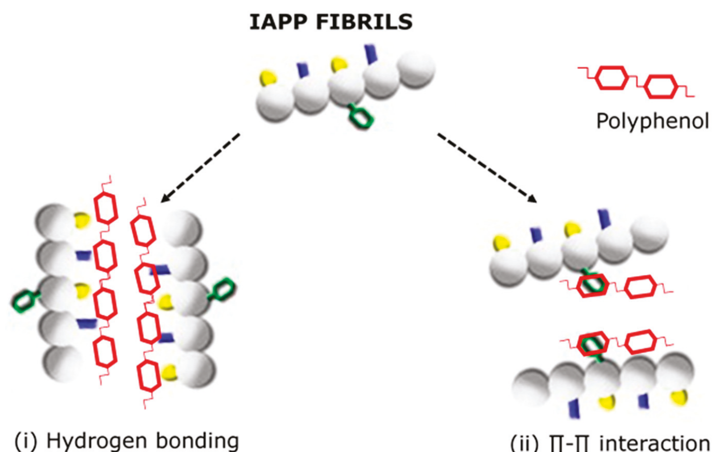


Figure 4. Proposed mechanisms by which polyphenols interfere with the self-assembly process of IAPP to inhibit the formation of cytotoxic oligomers (adapted from [131]).

Epigallocatechin gallate (EGCG), an important polyphenol found in green tea, has been shown to redirect amyloid fibril formation from fibrillogenic forms to non fibrillogenic oligomers; i.e., "off-pathway" aggregates that are unable to form amyloid [155,156] and have been shown to protect rat insulinoma, INS-1, cells from IAPP amyloid-induced cytotoxic effects [157]. EGCG binds to the native monomers to prevent their conversion into stable, β-sheet-rich structures, which are a prerequisite for nucleation-dependent amyloid fibril assembly, thus interfering with the early stages in the amyloid formation pathway. EGCG preferentially binds to the unfolded IAPP, due to the favourable spatial distribution of the poly-hydroxyl groups on the planar aromatic rings and its ability to form covalent bonds [12,158].

Similarly, resveratrol, a main constituent of grape seeds, has been shown to bind to both monomeric and fibrillar forms and to selectively remodel soluble oligomers and fibrillary intermediates to form less toxic oligomers of IAPP [13] in pancreatic β-cell line INS-1E [159,160]. The mechanism of inhibition, however, differs from that of EGCG. Simulation studies have demonstrated that resveratrol interferes with and blocks IAPP β-sheet side chain stacking [161], especially stacking of the aromatic rings, preventing the overall aggregation of the polypeptide. Additionally, oleuropeinaglycone (OLE), works in a similar manner to EGCG and resveratrol. OLE, the main phenolic component of olive oil, has been shown to interfere with the hIAPP fibrils to prevent the formation of toxic oligomers in RIN-5 F rat insulinoma cells [162]. The compound delays the conformational transition of hIAPP and redirects it to form off-pathway aggregates that are nontoxic. OLE also modulates the cytotoxic effects of the fibrils by preventing them from permeabilising the plasma membrane.

Curcumin, the main constituent of the rhizome *C. longa*, has been extensively investigated in in vitro and in vivo studies and has been shown to inhibit the formation of fibrils of IAPP in a concentration-dependent manner [13,163,164]. While curcumin has the ability to inhibit amyloid formation, it is unlikely that it can be used to prevent amyloidogenesis at ther in approximately apeutic concentrations in T2D. This is likely as curcumin is protective in INS cells against exogenous IAPP cytotoxicity within a narrow concentration range (10–25 μM); however, it is cytotoxic when concentration was increased above 25 μM [163]. A similar effect was shown using models of endogenous overexpression of hIAPP (INS cells and h-IAPP transgenic rat islets).

Rosamarinic acid, a phenolic derivative of caffeic acid found in many *Lamiaceae* herbs, has the ability to inhibit amyloid formation and destabilise preformed IAPP amyloid [164] by specifically binding to the polypeptide to inhibit its polymerisation. Additionally, polyphenolic molecules such as ferulic acid [165], a hydroxycinnamic acid; baicalein [165], a flavonoid found in the Chinese herb *Scutellariabaicalensis*; salvionolic acid B [166], a phenolic acid found in the Chinese herb *Salvia miltiorrhiza*; and silibinin [167], an active flavonoid constituent of silymarin, have been shown to inhibit the formation of an hIAPP amyloid β-sheet, preventing the aggregation of hIAPP fibrils and suppressing toxic oligomers of hIAPP monomers to reduce islet amyloid in vitro.

Again, recent work using myricetin [168,169] has found similar inhibitory effects on IAPP amyloid formation. Kao and colleagues [170] used the extracts of 13 fruits in vitro to analyse their ability to prevent the aggregation of amyloidogenic IAPP and found that flavonols from raspberries and blueberries were the strongest inhibitors of aggregation. Of the flavonols, quercetin and quercetin-3-*O*-rutinoside (rutin) have been shown to be potent inhibitors of IAPP aggregation and share structural similarities to tetracylines [91]. It is the aromatic rings of these bioactive molecules that have been proposed to competitively interact with residues of IAPP to prevent π–π interaction in order to inhibit the self-assembly process [92]. Alternatively, it has been suggested that the hydroxyl moieties of these flavonols inhibit fibril formation by creating hydrogen bonds in the amyloidogenic protein to modulate IAPP oligomerisation. The polycyclic nature of both quercitin and rutin interact with the amyloidogenic region in hA to suppress β-sheet formation and, therefore, have been shown to promote the formation of α-helix by hA, either by allowing its spontaneous formation or by promoting its formation from random coil [93].

It is important to consider that the absorption of dietary quercetin and rutin is largely determined by the chemical structure [81] and food matrix [171], which could limit digestion and metabolism [53], likely resulting in a lower bioavailability when compared to vitamin antioxidants [172]. Additionally, intestinal permeability [173] or co-administration with ascorbic acid [174] present in foods may influence the kinetics of absorption. Partial absorption occurs in the stomach and small intestine, the latter via hydrolysis by two different routes through the action of endogenous β-glucosidases—lactase phloridzin hydrolase (LPH) and cytosolic-β-glucosidase (CBG)—to generate more lipophilic, and thereby absorbable, aglycones [175]. While LPH is expressed at the brush border of enterocytes to selectively absorb quercetin glucoside [176], CBG hydrolyses conjugated glycosides that have been previously transported within enterocytes via the active sodium-dependent glucose transporter (SGLT1) [176–178]. Rutin, on the other hand, is not absorbed in the small intestine [179] and requires metabolism by colonic microflora [180]. Accordingly, in a comparative study, it was reported that quercetin glycosides are mostly absorbed in the stomach and small intestine, reaching peak serum concentration (C_{max}) in approximately 1.5 h, while absorption of rutin, which is dependent on the release of aglycones by the large bowel microbiota, reaches C_{max} later, in approximately 5.5 h [81]. These segmental differences in absorption are in line with reports by Hollman et al. [181–183], who showed that maximal absorption occurs at 0.5–0.7 h and 6–9 h following the ingestion of quercetin-4′-glucoside and rutin, respectively; hence the relatively low bioavailability of rutin (20%) compared to that of the glucoside moiety. Glycoside moiety is therefore a major determinant of the absorption of flavonoids to define biological activity and, in part, explains the varied responses from dietary intakes [184]. It is noteworthy that, prior to reaching portal circulation, these flavonoids can undergo phase II metabolism by methylation, sulfation and glucuronidation [56]. It is therefore pertinent to consider the levels of dietary intake to maintain physiological concentrations for novel mechanisms to be operational, which are difficult to achieve in plasma and extracellular fluids, with flavonoid concentrations reported in the micromolar range in these biological compartments [53]. Accordingly, intracellular levels have been estimated to reach picomolar or nanomolar concentrations [185]. Several approaches, including ingestion along with dietary fat [186,187], to promote the rafficking of quercetin and rutin across the gastrointestinal mucosa to increase bioaccumulation have been advocated [173,188]. Ergo, supplementation with 500 mg rutin

once daily for six weeks, a concentration equivalent to that used to ameliorate IAPP amyloid in human amylin transgenic (hAtg) mice [93], has been shown to increase circulating rutin or quercetin levels by at least 2.5 fold [189].

In vivo studies support the use of quercetin [94] and rutin [93] as antioxidants that additionally modulate IAPP aggregation, the latter using transgenic mice engineered to develop features of T2D in humans. This is in line with other studies that have shown that rutin interacts with soluble hA oligomers to prevent the formation of cytotoxic aggregates and to protect against Fas-mediated destruction of islet cells [8,91]. hAtg mice with β-cell specific IAPP expression [8] replicate the T2D phenotype of islet changes to develop diabetes [190], with rutin treatment shown to prolong the onset of diabetes and ameliorate the severity of diabetic syndrome in treated mice [93]. Hence, studies utilising human amylin transgenic (hAtg) models provide reliable critical mechanistic information [91,126,137] contributing toward our understanding of IAPP oligomerisation, its related cytotoxicity and resultant islet cell apoptosis. However, the beneficial effects of quercetin and rutin have not been evident in other studies [158]. Notably, it has been suggested that the rutinoside group, following ingestion of rutin, may be cleaved during intestinal transit to release quercetin and its glycosides, which could act as the bioactive compounds rather than rutin itself. However, this was disproved in a pharmacokinetic study that did not find free circulating quercetin in blood following dosage with rutin and quercetin-4-*O*-glucoside [191], possibly due to the degradation of rutin by colonic microflora into phenolic catabolites: 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid [192]. Again, the simultaneous detection of quercetin and rutin in plasma as well as lymph, following intra-duodenal administration in rats, seems to indicate that rutin may possibly be absorbed intact from intestinal cells [193]. This finding was in line with previous publications conducted in animal models [194,195]. While validation from human studies is certainly required, plasma quercetin has often been measured as a marker of rutin absorption to facilitate the ease of analyses [189]. While both the relative and absolute bioavailability of quercetin has been assessed, studies examining the former have most commonly been used in human studies.

4. Learnings from the Evidence and Concluding Remarks

T2D prevalence and its associated micro and macro-vascular risks continue to rise as a result of over-nutrition and lifestyle changes [196]. Accordingly, safer, natural and well-tolerated compounds, such as polyphenols that are widely available from dietary sources, with established antidiabetic effects, are emerging as novel therapeutic targets for delaying the progression and for preventing T2D in 'at-risk' individuals [63]. In addition to being anti-oxidant and anti-inflammatory agents [197], polyphenols exhibit anti-hyperglycaemic effects, as they improve carbohydrate metabolism, β-cell function and insulin resistance. This includes its novel role in arresting IAPP fibril formation and inhibiting the deleterious cytotoxic effects of IAPP amyloid.

Loss of functional β-cell mass is central to the pathophysiology of T2D [6]. IAPP misfolding and aggregation has recently emerged as a critical entity in islet cell pathology and T2D progression, with unequivocal data suggesting that the inhibition of the cytotoxic IAPP oligomers is key to improving pathology [97]. Whether these aggregates are a consequence of the tissue damage during disease progression or involved in disease pathogenesis remains to be determined. It is likely the latter, given that PMD is an established mechanism in the pathogenesis linked to the various neurodegenerative diseases [99]. On consideration, IAPP misfolding may play an important role in the transition from the prediabetic state to that of T2D and warrants further investigation.

Using polyphenol flavonoids to target amyloid deposits appears to be a rational, promising and novel therapeutic approach, given that the favourable phenolic ring structure and hydroxyl moieties allows them to function as potent inhibitors of IAPP oligomerisation. The benefits of using flavonoid polyphenols is that they occur naturally, have antioxidant properties, are stable in biological fluids, have the ability to cross the blood brain barrier and do not elicit an immune response. In addition, they have also been shown to inhibit 11β-HSD1 [152,153,198] and lower cortisol secretion. Dysregulated

cortisol secretion has been associated with the risk of T2D [199] and increased complications [200,201], especially greater diurnal secretion [202].

It is noteworthy that, although anti-oxidative properties of polyphenol flavonoids have been associated with the reduced risk of T2D, further research to elucidate the mechanism are required. ROS activity in in vitro studies has been observed at concentrations that are significantly higher than the physiological levels found in vivo [189]. Hence, while in vitro studies show positive effects, short term intervention studies are required to address and further elucidate the effects of these compounds in individuals diagnosed with prediabetes and T2D to address these proposed mechanisms of action. Discretion on the type and class of polyphenol, along with dosage, will be an important determinant of the outcomes, given the differences in bioavailability following dietary intake. Of the polyphenol flavonoids, the flavonol quercetin [90], the most abundantly present in the studied diet, and its rutinoside conjugate, rutin [93,203], may likely be most relevant to ameliorating the T2D risk, given that they are potent antioxidants [188], attenuate fasting and postprandial hyperglycaemia [204,205], and been shown to strongly inhibit IAPP-induced cytotoxicity.

Acknowledgments: IRS holds a postdoctoral fellowship funded by the High-Value Nutrition National Science Challenge and the Ministry for Business, Innovation and Employment (MBIE #3710040). SDP holds the Fonterra Chair in Human Nutrition at the University of Auckland, and receives funding from NZ Health Research Council, High-Value Nutrition National Science Challenge and the Ministry for Business, Innovation and Employment, Riddet centre of research excellence (CoRE).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Danaei, G.; Finucane, M.M.; Lu, Y.; Singh, G.M.; Cowan, M.J.; Paciorek, C.J.; Lin, J.K.; Farzadfar, F.; Khang, Y.-H.; Stevens, G.A.; et al. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: Systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2·7 million participants. *Lancet* **2011**, *378*, 31–40. [CrossRef]
2. Shaw, J.E.; Sicree, R.A.; Zimmet, P.Z. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res. Clin. Pract.* **2010**, *87*, 4–14. [CrossRef] [PubMed]
3. Ginter, E.; Simko, V. Global prevalence and future of diabetes mellitus. In *Diabetes: An Old Disease, a New Insigh*; Ahmad, S.I., Ed.; Springer: New York, NY, USA, 2013; pp. 35–41.
4. Sequeira, I.R.; Poppitt, S.D. Hb_{A1c} as a marker of prediabetes: A reliable screening tool or not? *Insights Nutr. Metab.* **2017**, *1*, 11–20.
5. Reaven, G.M. Role of insulin resistance in human disease. *Diabetes* **1988**, *37*, 1595–1607. [CrossRef] [PubMed]
6. Saisho, Y. Β-cell dysfunction: Its critical role in prevention and management of type 2 diabetes. *World J. Diabetes* **2015**, *6*, 109–124. [CrossRef] [PubMed]
7. Jaikaran, E.T.A.S.; Clark, A. Islet amyloid and type 2 diabetes: From molecular misfolding to islet pathophysiology. *Biochim. Biophys. Acta* **2001**, *1537*, 179–203. [CrossRef]
8. Zhang, S.; Liu, H.; Chuang, C.L.; Li, X.; Au, M.; Zhang, L.; Phillips, A.R.; Scott, D.W.; Cooper, G.J. The pathogenic mechanism of diabetes varies with the degree of overexpression and oligomerization of human amylin in the pancreatic islet β cells. *FASEB J.* **2014**, *28*, 5083–5096. [CrossRef] [PubMed]
9. Engel, M.F.; Khemtémourian, L.; Kleijer, C.C.; Meeldijk, H.J.; Jacobs, J.; Verkleij, A.J.; de Kruijff, B.; Killian, J.A.; Höppener, J.W. Membrane damage by human islet amyloid polypeptide through fibril growth at the membrane. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 6033–6038. [CrossRef] [PubMed]
10. Schubert, D.; Behl, C.; Lesley, R.; Brack, A.; Dargusch, R.; Sagara, Y.; Kimura, H. Amyloid peptides are toxic via a common oxidative mechanism. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 1989–1993. [CrossRef] [PubMed]
11. Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* **2001**, *414*, 813–820. [CrossRef] [PubMed]
12. Cao, P.; Raleigh, D.P. Analysis of the inhibition and remodeling of islet amyloid polypeptide amyloid fibers by flavanols. *Biochemistry* **2012**, *51*, 2670–2683. [CrossRef] [PubMed]

13. Nedumpully-Govindan, P.; Kakinen, A.; Pilkington, E.H.; Davis, T.P.; Ke, P.C.; Ding, F. Stabilizing off-pathway oligomers by polyphenol nanoassemblies for IAPP aggregation inhibition. *Sci. Rep.* **2016**, *6*, 19463. [CrossRef] [PubMed]
14. Sgarbossa, A. Natural biomolecules and protein aggregation: Emerging strategies against amyloidogenesis. *Int. J. Mol. Sci.* **2012**, *13*, 17121–17137. [CrossRef] [PubMed]
15. Hollenbeck, C.; Reaven, G.M. Variations in insulin-stimulated glucose uptake in healthy individuals with normal glucose tolerance. *J. Clin. Endocrinol. Metab.* **1987**, *64*, 1169–1173. [CrossRef] [PubMed]
16. Reaven, G.M.; Brand, R.J.; Chen, Y.D.; Mathur, A.K.; Goldfine, I. Insulin resistance and insulin secretion are determinants of oral glucose tolerance in normal individuals. *Diabetes* **1993**, *42*, 1324–1332. [CrossRef] [PubMed]
17. Reaven, G.; Hollenbeck, C.; Chen, Y.D. Relationship between glucose tolerance, insulin secretion, and insulin action in non-obese individuals with varying degrees of glucose tolerance. *Diabetologia* **1989**, *32*, 52–55. [CrossRef] [PubMed]
18. Collboration, N.R.F. Trends in adult body-mass index in 200 countries from 1975 to 2014: A pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet* **2016**, *387*, 1377–1396.
19. Ramachandran, A.; Wan Ma, R.C.; Snehalatha, C. Diabetes in Asia. *Lancet* **2010**, *375*, 408–418. [CrossRef]
20. Sattar, N.; Gill, J.M. Type 2 diabetes as a disease of ectopic fat? *BMC Med.* **2014**, *12*, 123. [CrossRef] [PubMed]
21. Astrup, A.; Finer, N. Redefining type 2 diabetes: 'Diabesity'or 'obesity dependent diabetes mellitus'? *Obes. Rev.* **2000**, *1*, 57–59. [CrossRef] [PubMed]
22. Popkin, B.M. Will China's nutrition transition overwhelm its health care system and slow economic growth? *Health Aff.* **2008**, *27*, 1064–1076. [CrossRef] [PubMed]
23. Mozaffarian, D.; Kamineni, A.; Carnethon, M.; Djoussé, L.; Mukamal, K.J.; Siscovick, D. Lifestyle risk factors and new-onset diabetes mellitus in older adults: The cardiovascular health study. *Arch. Intern. Med.* **2009**, *169*, 798–807. [CrossRef] [PubMed]
24. Dunkley, A.J.; Bodicoat, D.H.; Greaves, C.J.; Russell, C.; Yates, T.; Davies, M.J.; Khunti, K. Diabetes prevention in the real world: Effectiveness of pragmatic lifestyle interventions for the prevention of type 2 diabetes and of the impact of adherence to guideline recommendations. *Diabetes Care* **2014**, *37*, 922–933. [CrossRef] [PubMed]
25. Kim, S.; Popkin, B.M. Commentary: Understanding the epidemiology of overweight and obesity—A real global public health concern. *Int. J. Epidemiol.* **2006**, *35*, 60–67. [CrossRef] [PubMed]
26. Lindström, J.; Peltonen, M.; Eriksson, J.G.; Ilanne-Parikka, P.; Aunola, S.; Keinänen-Kiukaanniemi, S.; Uusitupa, M.; Tuomilehto, J.; Finnish Diabetes Prevention Study (DPS). Improved lifestyle and decreased diabetes risk over 13 years: Long-term follow-up of the randomised Finnish Diabetes Prevention Study (DPS). *Diabetologia* **2013**, *56*, 284–293. [CrossRef] [PubMed]
27. Ley, S.H.; Hamdy, O.; Mohan, V.; Hu, F.B. Prevention and management of type 2 diabetes: Dietary components and nutritional strategies. *Lancet* **2014**, *383*, 1999–2007. [CrossRef]
28. Li, G.; Zhang, P.; Wang, J.; Gregg, E.W.; Yang, W.; Gong, Q.; Li, H.; Li, H.; Jiang, Y.; An, Y.; et al. The long-term effect of lifestyle interventions to prevent diabetes in the China Da Qing diabetes prevention study: A 20-year follow-up study. *Lancet* **2008**, *371*, 1783–1789. [CrossRef]
29. Diabetes Prevention Program Research Group; Knowler, W.C.; Fowler, S.E.; Hamman, R.F.; Christophi, C.A.; Hoffman, H.J.; Brenneman, A.T.; Brown-Firday, J.O.; Goldberg, R.; Venditti, E.; et al. 10-year follow-up of diabetes incidence and weight loss in the diabetes prevention program outcomes study. *Lancet* **2009**, *374*, 1677–1686. [PubMed]
30. Ramachandran, A.; Snehalatha, C.; Mary, S.; Mukesh, B.; Bhaskar, A.D.; Vijay, V. The Indian Diabetes Prevention Programme shows that lifestyle modification and metformin prevent type 2 diabetes in Asian Indian subjects with impaired glucose tolerance (IDPP-1). *Diabetologia* **2006**, *49*, 289–297. [CrossRef] [PubMed]
31. Lindström, J.; Ilanne-Parikka, P.; Peltonen, M.; Aunola, S.; Eriksson, J.G.; Hemiö, K.; Hämäläinen, H.; Härkönen, P.; Keinänen-Kiukaanniemi, S.; Laakso, M.; et al. Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: Follow-up of the Finnish diabetes prevention study. *Lancet* **2006**, *368*, 1673–1679. [CrossRef]
32. Liu, A.Y.; Silvestre, M.P.; Poppitt, S.D. Prevention of type 2 diabetes through lifestyle modification: Is there a role for higher-protein diets? *Adv. Nutr.* **2015**, *6*, 665–673. [CrossRef] [PubMed]

33. Gillies, C.L.; Abrams, K.R.; Lambert, P.C.; Cooper, N.J.; Sutton, A.J.; Hsu, R.T.; Khunti, K. Pharmacological and lifestyle interventions to prevent or delay type 2 diabetes in people with impaired glucose tolerance: Systematic review and meta-analysis. *Br. Med. J.* **2007**, *334*, 299. [CrossRef] [PubMed]
34. Palmer, A.J.; Tucker, D.M.D. Cost and clinical implications of diabetes prevention in an Australian setting: A long-term modeling analysis. *Prim. Care Diabetes* **2012**, *6*, 109–121. [CrossRef] [PubMed]
35. Diabetes Prevention Program Research Group. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N. Engl. J. Med.* **2002**, *346*, 393–403.
36. Feskens, E.J.; Virtanen, S.M.; Räsänen, L.; Tuomilehto, J.; Stengård, J.; Pekkanen, J.; Nissinen, A.; Kromhout, D. Dietary factors determining diabetes and impaired glucose tolerance: A 20-year follow-up of the Finnish and Dutch cohorts of the seven countries study. *Diabetes Care* **1995**, *18*, 1104–1112. [CrossRef] [PubMed]
37. Villegas, R.; Shu, X.O.; Gao, Y.-T.; Yang, G.; Elasy, T.; Li, H.; Zheng, W. Vegetable but not fruit consumption reduces the risk of type 2 diabetes in Chinese women. *J. Nutr.* **2008**, *138*, 574–580. [PubMed]
38. Cooper, A.J.; Sharp, S.J.; Lentjes, M.A.H.; Luben, R.N.; Khaw, K.-T.; Wareham, N.J.; Forouhi, N.G. A prospective study of the association between quantity and variety of fruit and vegetable intake and incident type 2 diabetes. *Diabetes Care* **2012**, *35*, 1293–1300. [CrossRef] [PubMed]
39. Mursu, J.; Virtanen, J.K.; Tuomainen, T.-P.; Nurmi, T.; Voutilainen, S. Intake of fruit, berries, and vegetables and risk of type 2 diabetes in Finnish men: The Kuopio ischaemic heart disease risk factor study. *Am. J. Clin. Nutr.* **2014**, *99*, 328–333. [CrossRef] [PubMed]
40. Pérez-Jiménez, J.; Neveu, V.; Vos, F.; Scalbert, A. Systematic analysis of the content of 502 polyphenols in 452 foods and beverages: An application of the phenol-explorer database. *J. Agric. Food Chem.* **2010**, *58*, 4959–4969. [CrossRef] [PubMed]
41. Chun, O.K.; Chung, S.J.; Song, W.O. Estimated dietary flavonoid intake and major food sources of US adults. *J. Nutr.* **2007**, *137*, 1244–1252. [PubMed]
42. Zamora-Ros, R.; Knaze, V.; Rothwell, J.A.; Hémon, B.; Moskal, A.; Overvad, K.; Tjønneland, A.; Kyrø, C.; Fagherazzi, G.; Boutron-Ruault, M.C.; et al. Dietary polyphenol intake in Europe: The European prospective investigation into cancer and nutrition (EPIC) study. *Eur. J. Nutr.* **2016**, *55*, 1359–1375. [CrossRef] [PubMed]
43. Lindsay, D.G. The nutritional enhancement of plant foods in Europe 'NEODIET'. *Trends Food Sci. Technol.* **2000**, *11*, 145–151. [CrossRef]
44. Clifford, M. Diet-derived phenols in plasma and tissues and their implications for health. *Planta Med.* **2004**, *70*, 1103–1114. [CrossRef] [PubMed]
45. Magrone, T.; Perez de Heredia, F.; Jirillo, E.; Morabito, G.; Marcos, A.; Serafini, M. Functional foods and nutraceuticals as therapeutic tools for the treatment of diet-related diseases. *Can. J. Physiol. Pharmacol.* **2013**, *91*, 387–396. [CrossRef] [PubMed]
46. Hanhineva, K.; Törrönen, R.; Bondia-Pons, I.; Pekkinen, J.; Kolehmainen, M.; Mykkänen, H.; Poutanen, K. Impact of dietary polyphenols on carbohydrate metabolism. *Int. J. Mol. Sci.* **2010**, *11*, 1365–1402. [CrossRef] [PubMed]
47. Sun, Q.; Wedick, N.M.; Tworoger, S.S.; Pan, A.; Townsend, M.K.; Cassidy, A.; Franke, A.A.; Rimm, E.B.; Hu, F.B.; van Dam, R.M. Urinary excretion of select dietary polyphenol metabolites is associated with a lower risk of type 2 diabetes in proximate but not remote follow-up in a prospective investigation in 2 cohorts of US women. *J. Nutr.* **2015**, *145*, 1280–1288. [CrossRef] [PubMed]
48. Zamora-Ros, R.; Forouhi, N.G.; Sharp, S.J.; González, C.A.; Buijsse, B.; Guevara, M.; van der Schouw, Y.T.; Amiano, P.; Boeing, H.; Bredsdorff, L.; et al. The association between dietary flavonoid and lignan intakes and incident type 2 diabetes in European populations: The EPIC-interact study. *Diabetes Care* **2013**, *36*, 3961–3970. [CrossRef] [PubMed]
49. Wedick, N.M.; Pan, A.; Cassidy, A.; Rimm, E.B.; Sampson, L.; Rosner, B.; Willett, W.; Hu, F.B.; Sun, Q.; van Dam, R.M. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. *Am. J. Clin. Nutr.* **2012**, *95*, 925–933. [CrossRef] [PubMed]
50. Song, Y.; Manson, J.E.; Buring, J.E.; Sesso, H.D.; Liu, S. Associations of dietary flavonoids with risk of type 2 diabetes, and, markers of insulin resistance and systemic inflammation in women: A prospective study and cross-sectional analysis. *J. Am. Coll. Nutr.* **2005**, *24*, 376–384. [CrossRef] [PubMed]
51. Knekt, P.; Kumpulainen, J.; Järvinen, R.; Rissanen, H.; Heliövaara, M.; Reunanen, A.; Hakulinen, T.; Aromaa, A. Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* **2002**, *76*, 560–568. [PubMed]

52. Nettleton, J.A.; Harnack, L.J.; Scrafford, C.G.; Mink, P.J.; Barraj, L.M.; Jacobs, D.R. Dietary flavonoids and flavonoid-rich foods are not associated with risk of type 2 diabetes in postmenopausal women. *J. Nutr.* **2006**, *136*, 3039–3045. [PubMed]
53. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S. [PubMed]
54. Spencer, J.P.; El Mohsen, M.M.A.; Minihane, A.-M.; Mathers, J.C. Biomarkers of the intake of dietary polyphenols: Strengths, limitations and application in nutrition research. *Br. J. Nutr.* **2008**, *99*, 12–22. [CrossRef] [PubMed]
55. Takechi, R.; Alfonso, H.; Harrison, A.; Hiramatsu, N.; Ishisaka, A.; Tanaka, A.; Tan, L.B.; Lee, A.H. Assessing self-reported green tea and coffee consumption by food frequency questionnaire and food record and their association with polyphenol biomarkers in Japanese women. *Asia Pac. J. Clin. Nutr.* **2017**. [CrossRef]
56. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892. [CrossRef] [PubMed]
57. Rodriguez-Mateos, A.; Vauzour, D.; Krueger, C.G.; Shanmuganayagam, D.; Reed, J.; Calani, L.; Mena, P.; Del Rio, D.; Crozier, A. Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: An update. *Arch. Toxicol.* **2014**, *88*, 1803–1853. [CrossRef] [PubMed]
58. Zanotti, I.; Dall'Asta, M.; Mena, P.; Mele, L.; Bruni, R.; Ray, S.; Del Rio, D. Atheroprotective effects of (poly) phenols: A focus on cell cholesterol metabolism. *Food Funct.* **2015**, *6*, 13–31. [CrossRef] [PubMed]
59. Hughes, L.A.; Arts, I.C.; Ambergen, T.; Brants, H.A.; Dagnelie, P.C.; Goldbohm, R.A.; van den Brandt, P.A.; Weijenberg, M.P. Higher dietary flavone, flavonol, and catechin intakes are associated with less of an increase in BMI over time in women: A longitudinal analysis from the Netherlands cohort study. *Am. J. Clin. Nutr.* **2008**, *88*, 1341–1352. [PubMed]
60. Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287–306. [CrossRef] [PubMed]
61. Liu, Y.-J.; Zhan, J.; Liu, X.-L.; Wang, Y.; Ji, J.; He, Q.-Q. Dietary flavonoids intake and risk of type 2 diabetes: A meta-analysis of prospective cohort studies. *Clin. Nutr.* **2014**, *33*, 59–63. [CrossRef] [PubMed]
62. Anhê, F.F.; Desjardins, Y.; Pilon, G.; Dudonné, S.; Genovese, M.I.; Lajolo, F.M.; Marette, A. Polyphenols and type 2 diabetes: A prospective review. *PharmaNutrition* **2013**, *1*, 105–114. [CrossRef]
63. Bahadoran, Z.; Mirmiran, P.; Azizi, F. Dietary polyphenols as potential nutraceuticals in management of diabetes: A review. *J. Diabetes Metab. Disord.* **2013**, *12*, 43. [CrossRef] [PubMed]
64. Xiao, J.; Hogger, P. Dietary polyphenols and type 2 diabetes: Current insights and future perspectives. *Curr. Med. Chem.* **2015**, *22*, 23–38. [CrossRef] [PubMed]
65. George, A.S.; Ben, A.C.; Efthimios, K.; Anastassia, L.K.; Demetra, S.M.C.; Vassiliki, T.S.; Atsushi, K.; Joseph, M.H.; Demetres, D.L. Phytogenic polyphenols as glycogen phosphorylase inhibitors: The potential of triterpenes and flavonoids for glycaemic control in type 2 diabetes. *Curr. Med. Chem.* **2017**, *24*, 384–403.
66. Abunab, H.; Dator, W.L.; Hawamdeh, S. Effect of olive leaf extract on glucose levels in diabetes-induced rats: A systematic review and meta-analysis. *J. Diabetes* **2016**. [CrossRef] [PubMed]
67. De Bock, M.; Derraik, J.G.; Brennan, C.M.; Biggs, J.B.; Morgan, P.E.; Hodgkinson, S.C.; Hofman, P.L.; Cutfield, W.S. Olive (*Olea europaea* L.) leaf polyphenols improve insulin sensitivity in middle-aged overweight men: A randomized, placebo-controlled, crossover trial. *PLoS ONE* **2013**, *8*, e57622. [CrossRef] [PubMed]
68. Wainstein, J.; Ganz, T.; Boaz, M.; Bar Dayan, Y.; Dolev, E.; Kerem, Z.; Madar, Z. Olive leaf extract as a hypoglycemic agent in both human diabetic subjects and in rats. *J. Med. Food* **2012**, *15*, 605–610. [CrossRef] [PubMed]
69. Boaz, M.; Leibovitz, E.; Dayan, Y.B.; Wainstein, J. Functional foods in the treatment of type 2 diabetes: Olive leaf extract, turmeric and fenugreek, a qualitative review. *Funct. Foods Health Dis.* **2011**, *1*, 472–481.
70. Cumaoglu, A.; Rackova, L.; Stefek, M.; Kartal, M.; Maechler, P.; Karasu, Ç. Effects of olive leaf polyphenols against H_2O_2 toxicity in insulin secreting β-cells. *Acta Biochim. Pol.* **2011**, *58*, 45–50. [PubMed]
71. Palma-Duran, S.A.; Vlassopoulos, A.; Lean, M.; Govan, L.; Combet, E. Nutritional intervention and impact of polyphenol on glycohemoglobin (HbA1c) in non-diabetic and type 2 diabetic subjects: Systematic review and meta-analysis. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 975–986. [CrossRef] [PubMed]
72. Stevenson, D.E.; Hurst, R.D. Polyphenolic phytochemicals—Just antioxidants or much more? *Cell. Mol. Life Sci.* **2007**, *64*, 2900–2916. [CrossRef] [PubMed]

73. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*, 1231–1246. [CrossRef] [PubMed]
74. Hollman, P.C.H.; van Trijp, J.M.P.; Buysman, M.N.C.P.; van der Gaag, M.S.; Mengelers, M.J.B.; de Vries, J.H.M.; Katan, M.B. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.* **1997**, *418*, 152–156. [CrossRef]
75. Bhagwat, S.; Haytowitz, D.B.; Holden, J.M. *Usda Database for the Flavonoid Content of Selected Foods, Release 3.1*; US Department of Agriculture: Beltsville, MD, USA, 2014.
76. Kreft, I.; Fabjan, N.; Yasumoto, K. Rutin content in buckwheat (fagopyrum esculentum moench) food materials and products. *Food Chem.* **2006**, *98*, 508–512. [CrossRef]
77. Iacopini, P.; Baldi, M.; Storchi, P.; Sebastiani, L. Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, in vitro antioxidant activity and interactions. *J. Food Compos. Anal.* **2008**, *21*, 589–598. [CrossRef]
78. Sun, T.; Powers, J.R.; Tang, J. Evaluation of the antioxidant activity of asparagus, broccoli and their juices. *Food Chem.* **2007**, *105*, 101–106. [CrossRef]
79. Bajpai, M.; Mishra, A.; Prakash, D. Antioxidant and free radical scavenging activities of some leafy vegetables. *Int. J. Food Sci. Nutr.* **2005**, *56*, 473–481. [CrossRef] [PubMed]
80. Şahin, S. Evaluation of antioxidant properties and phenolic composition of fruit tea infusions. *Antioxidants* **2013**, *2*, 206–215. [CrossRef] [PubMed]
81. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S. [PubMed]
82. Kühnau, J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. In *World Review of Nutrition and Dietetics*; Karger Publishers: Basel, Switzerland, 1976; Volume 24, pp. 117–191.
83. Lachman, J.; Orsak, M.; Pivec, V.; Faustusova, E. Content of rutin in selected plant sources. *Sci. Agric. Bohem.* **2000**, *31*, 89–99.
84. Jiang, P.; Burczynski, F.; Campbell, C.; Pierce, G.; Austria, J.A.; Briggs, C.J. Rutin and flavonoid contents in three buckwheat species *Fagopyrum esculentum*, *F. tataricum*, and *F. homotropicum* and their protective effects against lipid peroxidation. *Food Res. Int.* **2007**, *40*, 356–364. [CrossRef]
85. Li, Y.Q.; Zhou, F.C.; Gao, F.; Bian, J.S.; Shan, F. Comparative evaluation of quercetin, isoquercetin and rutin as inhibitors of α-glucosidase. *J. Agric. Food Chem.* **2009**, *57*, 11463–11468. [CrossRef] [PubMed]
86. Jadhav, R.; Puchchakayala, G. Hypoglycemic and antidiabetic activity of flavonoids: Boswellic acid, ellagic acid, quercetin, rutin on streptozotocin-nicotinamide induced type 2 diabetic rats. *Int. J. Pharm. Pharm. Sci.* **2012**, *1*, 251–256.
87. Kamalakkannan, N.; Prince, P.S.M. Antihyperglycaemic and antioxidant effect of rutin, a polyphenolic flavonoid, in streptozotocin-induced diabetic wistar rats. *Basic Clin. Pharmacol. Toxicol.* **2006**, *98*, 97–103. [CrossRef] [PubMed]
88. Jeong, S.M.; Kang, M.J.; Choi, H.N.; Kim, J.H.; Kim, J.I. Quercetin ameliorates hyperglycemia and dyslipidemia and improves antioxidant status in type 2 diabetic db/db mice. *Nutr. Res. Pract.* **2012**, *6*, 201–207. [CrossRef] [PubMed]
89. Coskun, O.; Kanter, M.; Korkmaz, A.; Oter, S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas. *Pharmacol. Res.* **2005**, *51*, 117–123. [CrossRef] [PubMed]
90. Alam, M.M.; Meerza, D.; Naseem, I. Protective effect of quercetin on hyperglycemia, oxidative stress and DNA damage in alloxan induced type 2 diabetic mice. *Life Sci.* **2014**, *109*, 8–14. [CrossRef] [PubMed]
91. Aitken, J.F.; Loomes, K.M.; Scott, D.W.; Reddy, S.; Phillips, A.R.; Prijic, G.; Fernando, C.; Zhang, S.; Broadhurst, R.; L'huillier, P.; et al. Tetracycline treatment retards the onset and slows the progression of diabetes in human amylin/islet amyloid polypeptide transgenic mice. *Diabetes* **2010**, *59*, 161–171. [CrossRef] [PubMed]
92. Ahmad, E.; Ahmad, A.; Singh, S.; Arshad, M.; Khan, A.H.; Khan, R.H. A mechanistic approach for islet amyloid polypeptide aggregation to develop anti-amyloidogenic agents for type-2 diabetes. *Biochimie* **2011**, *93*, 793–805. [CrossRef] [PubMed]

93. Aitken, J.F.; Loomes, K.M.; Riba-Garcia, I.; Unwin, R.D.; Prijic, G.; Phillips, A.S.; Phillips, A.R.; Wu, D.; Poppitt, S.D.; Ding, K.; et al. Rutin suppresses human-amylin/hIAPP misfolding and oligomer formation in vitro, and ameliorates diabetes and its impacts in human-amylin/hIAPP transgenic mice. *Biochem. Biophys. Res. Commun.* **2017**, *482*, 625–631. [CrossRef] [PubMed]
94. López, L.; Varea, O.; Navarro, S.; Carrodeguas, J.; Sanchez de Groot, N.; Ventura, S.; Sancho, J. Benzbromarone, quercetin, and folic acid inhibit amylin aggregation. *Int. J. Mol. Sci.* **2016**, *17*, 964. [CrossRef] [PubMed]
95. Cooper, G.; Leighton, B.; Dimitriadis, G.; Parry-Billings, M.; Kowalchuk, J.; Howland, K.; Rothbard, J.; Willis, A.; Reid, K. Amylin found in amyloid deposits in human type 2 diabetes mellitus may be a hormone that regulates glycogen metabolism in skeletal muscle. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7763–7766. [CrossRef] [PubMed]
96. Haataja, L.; Gurlo, T.; Huang, C.J.; Butler, P.C. Islet amyloid in type 2 diabetes, and the toxic oligomer hypothesis. *Endocr. Rev.* **2008**, *29*, 303–316. [CrossRef] [PubMed]
97. Hull, R.L.; Westermark, G.T.; Westermark, P.; Kahn, S.E. Islet amyloid: A critical entity in the pathogenesis of type 2 diabetes. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 3629–3643. [CrossRef] [PubMed]
98. Konarkowska, B.; Aitken, J.F.; Kistler, J.; Zhang, S.; Cooper, G.J. The aggregation potential of human amylin determines its cytotoxicity towards islet β-cells. *FEBS J.* **2006**, *273*, 3614–3624. [CrossRef] [PubMed]
99. Soto, C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat. Rev. Neurosci.* **2003**, *4*, 49–60. [CrossRef] [PubMed]
100. Westermark, P.; Wernstedt, C.; Wilander, E.; Hayden, D.W.; O'Brien, T.D.; Johnson, K.H. Amyloid fibrils in human insulinoma and islets of langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 3881–3885. [CrossRef] [PubMed]
101. Gedulin, B.R.; Jodka, C.M.; Herrmann, K.; Young, A.A. Role of endogenous amylin in glucagon secretion and gastric emptying in rats demonstrated with the selective antagonist, ac187. *Regul. Pept.* **2006**, *137*, 121–127. [CrossRef] [PubMed]
102. Wookey, P.J.; Lutz, T.A.; Andrikopoulos, S. Amylin in the periphery II: An updated mini-review. *Sci. World J.* **2006**, *6*, 1641–1655. [CrossRef] [PubMed]
103. Mukherjee, A.; Morales-Scheihing, D.; Butler, P.C.; Soto, C. Type 2 diabetes as a protein misfolding disease. *Trends Mol. Med.* **2015**, *21*, 439–449. [CrossRef] [PubMed]
104. Rafacho, A.; Ortsäter, H.; Nadal, A.; Quesada, I. Glucocorticoid treatment and endocrine pancreas function: Implications for glucose homeostasis, insulin resistance and diabetes. *J. Endocrinol.* **2014**, *223*, R49–R62. [CrossRef] [PubMed]
105. Green, K.N.; Billings, L.M.; Roozendaal, B.; McGaugh, J.L.; LaFerla, F.M. Glucocorticoids increase amyloid-β and tau pathology in a mouse model of Alzheimer's disease. *J. Neurosci.* **2006**, *26*, 9047–9056. [CrossRef] [PubMed]
106. Bretherton-Watt, D.; Ghatei, M.; Bloom, S.; Jamal, H.; Ferrier, G.J.; Girgis, S.; Legon, S. Altered islet amyloid polypeptide (amylin) gene expression in rat models of diabetes. *Diabetologia* **1989**, *32*, 881–883. [CrossRef] [PubMed]
107. Koranyi, L.; Bourey, R.; Turk, J.; Mueckler, M.; Permutt, M. Differential expression of rat pancreatic islet beta-cell glucose transporter (GLUT 2), proinsulin and islet amyloid polypeptide genes after prolonged fasting, insulin-induced hypoglycaemia and dexamethasone treatment. *Diabetologia* **1992**, *35*, 1125–1132. [CrossRef] [PubMed]
108. Pieber, T.R.; Stein, D.T.; Ogawa, A.; Alam, T.; Ohneda, M.; McCorkle, K.; Chen, L.; McGarry, J.; Unger, R. Amylin-insulin relationships in insulin resistance with and without diabetic hyperglycemia. *Am. J. Physiol.-Endocrinol. Metab.* **1993**, *265*, E446–E453.
109. Ludvik, B.; Clodi, M.; Kautzky-Willer, A.; Capek, M.; Hartter, E.; Pacini, G.; Prager, R. Effect of dexamethasone on insulin sensitivity, islet amyloid polypeptide and insulin secretion in humans. *Diabetologia* **1993**, *36*, 84–87. [CrossRef] [PubMed]
110. Anagnostis, P.; Katsiki, N.; Adamidou, F.; Athyros, V.G.; Karagiannis, A.; Kita, M.; Mikhailidis, D.P. 11β-hydroxysteroid dehydrogenase type 1 inhibitors: Novel agents for the treatment of metabolic syndrome and obesity-related disorders? *Metabolism* **2013**, *62*, 21–33. [CrossRef] [PubMed]

111. Morgan, S.A.; Sherlock, M.; Gathercole, L.L.; Lavery, G.G.; Lenaghan, C.; Bujalska, I.J.; Laber, D.; Yu, A.; Convey, G.; Mayers, R.; et al. 11β-hydroxysteroid dehydrogenase type 1 regulates glucocorticoid-induced insulin resistance in skeletal muscle. *Diabetes* **2009**, *58*, 2506–2515. [CrossRef] [PubMed]
112. Masuzaki, H.; Flier, J.S. Tissue-specific glucocorticoid reactivating enzyme, 11β-hydroxysteoid dehydrogenase type 1 (11β-HSD1)-a promising drug target for the treatment of metabolic syndrome. *Curr. Drug Targets-Immune Endocr. Metab. Disord.* **2003**, *3*, 255–262. [CrossRef]
113. Tomlinson, J.W.; Finney, J.; Gay, C.; Hughes, B.A.; Hughes, S.V.; Stewart, P.M. Impaired glucose tolerance and insulin resistance are associated with increased adipose 11β-hydroxysteroid dehydrogenase type 1 expression and elevated hepatic 5α-reductase activity. *Diabetes* **2008**, *57*, 2652–2660. [CrossRef] [PubMed]
114. Van Raalte, D.; Ouwens, D.; Diamant, M. Novel insights into glucocorticoid-mediated diabetogenic effects: Towards expansion of therapeutic options? *Eur. J. Clin. Investig.* **2009**, *39*, 81–93. [CrossRef] [PubMed]
115. Opie, E.L. The relation of diabetes mellitus to lesions of the pancreas. Hyaline degeneration of the islands of Langerhans. *J. Exp. Med.* **1901**, *5*, 527–540. [CrossRef] [PubMed]
116. Westermark, P. Quantitative studies of amyloid in the islets of Langerhans. *Ups. J. Med. Sci.* **1972**, *77*, 91–94. [CrossRef] [PubMed]
117. Clark, A.; Wells, C.; Buley, I.; Cruickshank, J.; Vanhegan, R.; Matthews, D.; Cooper, G.J.; Holman, R.; Turner, R. Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: Quantitative changes in the pancreas in type 2 diabetes. *Diabetes Res.* **1988**, *9*, 151–159. [PubMed]
118. Jurgens, C.A.; Toukatly, M.N.; Fligner, C.L.; Udayasankar, J.; Subramanian, S.L.; Zraika, S.; Aston-Mourney, K.; Carr, D.B.; Westermark, P.; Westermark, G.T.; et al. β-cell loss and β-cell apoptosis in human type 2 diabetes are related to islet amyloid deposition. *Am. J. Pathol.* **2011**, *178*, 2632–2640. [CrossRef] [PubMed]
119. Westermark, P.; Wilander, E. The influence of amyloid deposits on the islet volume in maturity onset diabetes mellitus. *Diabetologia* **1978**, *15*, 417–421. [CrossRef] [PubMed]
120. Pike, K.E.; Savage, G.; Villemagne, V.L.; Ng, S.; Moss, S.A.; Maruff, P.; Mathis, C.A.; Klunk, W.E.; Masters, C.L.; Rowe, C.C. β-amyloid imaging and memory in non-demented individuals: Evidence for preclinical alzheimer's disease. *Brain* **2007**, *130*, 2837–2844. [CrossRef] [PubMed]
121. Chételat, G.; La Joie, R.; Villain, N.; Perrotin, A.; de La Sayette, V.; Eustache, F.; Vandenberghe, R. Amyloid imaging in cognitively normal individuals, at-risk populations and preclinical Alzheimer's disease. *NeuroImage Clin.* **2013**, *2*, 356–365. [CrossRef] [PubMed]
122. Howard, C.F. Longitudinal studies on the development of diabetes in individual Macaca nigra. *Diabetologia* **1986**, *29*, 301–306. [CrossRef] [PubMed]
123. Guardado-Mendoza, R.; Davalli, A.M.; Chavez, A.O.; Hubbard, G.B.; Dick, E.J.; Majluf-Cruz, A.; Tene-Perez, C.E.; Goldschmidt, L.; Hart, J.; Perego, C.; et al. Pancreatic islet amyloidosis, β-cell apoptosis, and α-cell proliferation are determinants of islet remodeling in type-2 diabetic baboons. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 13992–13997. [CrossRef] [PubMed]
124. Janson, J.E.; Soeller, W.C.; Roche, P.C.; Nelson, R.T.; Torchia, A.J.; Kreutter, D.K.; Butler, P.C. Spontaneous diabetes mellitus in transgenic mice expressing human islet amyloid polypeptide. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 7283–7288. [CrossRef] [PubMed]
125. Butler, A.E.; Janson, J.; Bonner-Weir, S.; Ritzel, R.; Rizza, R.A.; Butler, P.C. β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes. *Diabetes* **2003**, *52*, 102–110. [CrossRef] [PubMed]
126. Zhang, S.; Liu, H.; Yu, H.; Cooper, G.J. Fas-associated death receptor signaling evoked by human amylin in islet β-cells. *Diabetes* **2008**, *57*, 348–356. [CrossRef] [PubMed]
127. Gurlo, T.; Ryazantsev, S.; Huang, C.-J.; Yeh, M.W.; Reber, H.A.; Hines, O.J.; O'Brien, T.D.; Glabe, C.G.; Butler, P.C. Evidence for proteotoxicity in β cells in type 2 diabetes: Toxic islet amyloid polypeptide oligomers form intracellularly in the secretory pathway. *Am. J. Pathol.* **2010**, *176*, 861–869. [CrossRef] [PubMed]
128. Green, J.D.; Goldsbury, C.; Kistler, J.; Cooper, G.J.; Aebi, U. Human amylin oligomer growth and fibril elongation define two distinct phases in amyloid formation. *J. Biol. Chem.* **2004**, *279*, 12206–12212. [CrossRef] [PubMed]
129. Sindelar, C.V.; Hendsch, Z.S.; Tidor, B. Effects of salt bridges on protein structure and design. *Protein Sci.* **1998**, *7*, 1898–1914. [CrossRef] [PubMed]

130. Gazit, E. A possible role for π-stacking in the self-assembly of amyloid fibrils. *FASEB J.* **2002**, *16*, 77–83. [CrossRef] [PubMed]
131. Cheng, B.; Gong, H.; Xiao, H.; Petersen, R.B.; Zheng, L.; Huang, K. Inhibiting toxic aggregation of amyloidogenic proteins: A therapeutic strategy for protein misfolding diseases. *Biochim. Biophys. Acta* **2013**, *1830*, 4860–4871. [CrossRef] [PubMed]
132. Butler, A.E.; Janson, J.; Soeller, W.C.; Butler, P.C. Increased β-cell apoptosis prevents adaptive increase in β-cell mass in mouse model of type 2 diabetes: Evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes* **2003**, *52*, 2304–2314. [CrossRef] [PubMed]
133. Janson, J.; Ashley, R.H.; Harrison, D.; McIntyre, S.; Butler, P.C. The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles. *Diabetes* **1999**, *48*, 491–498. [CrossRef] [PubMed]
134. Huang, C.J.; Gurlo, T.; Haataja, L.; Costes, S.; Daval, M.; Ryazantsev, S.; Wu, X.; Butler, A.E.; Butler, P.C. Calcium-activated calpain-2 is a mediator of beta cell dysfunction and apoptosis in type 2 diabetes. *J. Biol. Chem.* **2010**, *285*, 339–348. [CrossRef] [PubMed]
135. Mirzabekov, T.A.; Lin, M.-C.; Kagan, B.L. Pore formation by the cytotoxic islet amyloid peptide amylin. *J. Biol. Chem.* **1996**, *271*, 1988–1992. [CrossRef] [PubMed]
136. Zhang, S.; Liu, J.; MacGibbon, G.; Dragunow, M.; Cooper, G.J. Increased expression and activation of c-jun contributes to human amylin-induced apoptosis in pancreatic islet β-cells. *J. Mol. Biol.* **2002**, *324*, 271–285. [CrossRef]
137. Zhang, S.; Liu, J.; Dragunow, M.; Cooper, G.J. Fibrillogenic amylin evokes islet β-cell apoptosis through linked activation of a caspase cascade and JNK1. *J. Biol. Chem.* **2003**, *278*, 52810–52819. [CrossRef] [PubMed]
138. Tucker, H.M.; Rydel, R.E.; Wright, S.; Estus, S. Human amylin induces "apoptotic" pattern of gene expression concomitant with cortical neuronal apoptosis. *J. Neurochem.* **1998**, *71*, 506–516. [CrossRef] [PubMed]
139. Saafi, E.L.; Konarkowska, B.; Zhang, S.; Kistler, J.; Cooper, G.J. Ultrastructural evidence that apoptosis is the mechanism by which human amylin evokes death in RINm5F pancreatic islet β-cells. *Cell Biol. Int.* **2001**, *25*, 339–350. [CrossRef] [PubMed]
140. Gillmore, J.D.; Hawkins, P.N.; Pepys, M.B. Amyloidosis: A review of recent diagnostic and therapeutic developments. *Br. J. Haematol.* **1997**, *99*, 245–256. [CrossRef] [PubMed]
141. Ahrén, B.; Oosterwijk, C.; Lips, C.; Höppener, J. Transgenic overexpression of human islet amyloid polypeptide inhibits insulin secretion and glucose elimination after gastric glucose gavage in mice. *Diabetologia* **1998**, *41*, 1374–1380. [CrossRef] [PubMed]
142. Gebre-Medhin, S.; Mulder, H.; Pekny, M.; Westermark, G.; Törnell, J.; Westermark, P.; Sundler, F.; Ahrén, B.; Betsholtz, C. Increased insulin secretion and glucose tolerance in mice lacking islet amyloid polypeptide (amylin). *Biochem. Biophys. Res. Commun.* **1998**, *250*, 271–277. [CrossRef] [PubMed]
143. Kulkarni, R.; Smith, D.; Ghatei, M.; Jones, P.; Bloom, S. Investigation of the effects of antisense oligodeoxynucleotides to islet amyloid polypeptide mrna on insulin release, content and expression. *J. Endocrinol.* **1996**, *151*, 341–348. [CrossRef] [PubMed]
144. Novials, A.; Jiménez-Chillarón, J.C.; Franco, C.; Casamitjana, R.; Gomis, R.; Gómez-Foix, A.M. Reduction of islet amylin expression and basal secretion by adenovirus-mediated delivery of amylin antisense cdna. *Pancreas* **1998**, *17*, 182–186. [CrossRef] [PubMed]
145. Lindström, T.; Leckström, A.; Westermark, P.; Arnqvist, H. Effect of insulin treatment on circulating islet amyloid polypeptide in patients with NIDDM. *Diabetic Med.* **1997**, *14*, 472–476. [CrossRef]
146. Soto, C.; Sigurdsson, E.M.; Morelli, L.; Kumar, R.A.; Castaño, E.M.; Frangione, B. β-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: Implications for alzheimer's therapy. *Nat. Med.* **1998**, *4*, 822–826. [CrossRef] [PubMed]
147. Nedumpully-Govindan, P.; Ding, F. Inhibition of IAPP aggregation by insulin depends on the insulin oligomeric state regulated by zinc ion concentration. *Sci. Rep.* **2015**, *5*, 8240. [CrossRef] [PubMed]
148. Bieschke, J.; Herbst, M.; Wiglenda, T.; Friedrich, R.P.; Boeddrich, A.; Schiele, F.; Kleckers, D.; del Amo, J.M.L.; Grüning, B.A.; Wang, Q.; et al. Small-molecule conversion of toxic oligomers to nontoxic β-sheet–rich amyloid fibrils. *Nat. Chem. Biol.* **2012**, *8*, 93–101. [CrossRef] [PubMed]
149. Forloni, G.; Colombo, L.; Girola, L.; Tagliavini, F.; Salmona, M. Anti-amyloidogenic activity of tetracyclines: Studies in vitro. *FEBS Lett.* **2001**, *487*, 404–407. [CrossRef]

150. Stoilova, T.; Colombo, L.; Forloni, G.; Tagliavini, F.; Salmona, M. A new face for old antibiotics: Tetracyclines in treatment of amyloidoses. *J. Med. Chem.* **2013**, *56*, 5987–6006. [CrossRef] [PubMed]
151. Wu, C.; Lei, H.; Wang, Z.; Zhang, W.; Duan, Y. Phenol red interacts with the protofibril-like oligomers of an amyloidogenic hexapeptide NFGAIL through both hydrophobic and aromatic contacts. *Biophys. J.* **2006**, *91*, 3664–3672. [CrossRef] [PubMed]
152. Torres-Piedra, M.; Ortiz-Andrade, R.; Villalobos-Molina, R.; Singh, N.; Medina-Franco, J.L.; Webster, S.P.; Binnie, M.; Navarrete-Vázquez, G.; Estrada-Soto, S. A comparative study of flavonoid analogues on streptozotocin–nicotinamide induced diabetic rats: Quercetin as a potential antidiabetic agent acting via 11β-hydroxysteroid dehydrogenase type 1 inhibition. *Eur. J. Med. Chem.* **2010**, *45*, 2606–2612. [CrossRef] [PubMed]
153. Hintzpeter, J.; Stapelfeld, C.; Loerz, C.; Martin, H.-J.; Maser, E. Green tea and one of its constituents, epigallocatechine-3-gallate, are potent inhibitors of human 11β-hydroxysteroid dehydrogenase type 1. *PLoS ONE* **2014**, *9*, e84468. [CrossRef] [PubMed]
154. Ladiwala, A.R.A.; Dordick, J.S.; Tessier, P.M. Aromatic small molecules remodel toxic soluble oligomers of amyloid β through three independent pathways. *J. Biol. Chem.* **2011**, *286*, 3209–3218. [CrossRef] [PubMed]
155. Bieschke, J.; Russ, J.; Friedrich, R.P.; Ehrnhoefer, D.E.; Wobst, H.; Neugebauer, K.; Wanker, E.E. EGCG remodels mature α-synuclein and amyloid-β fibrils and reduces cellular toxicity. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 7710–7715. [CrossRef] [PubMed]
156. Young, L.M.; Cao, P.; Raleigh, D.P.; Ashcroft, A.E.; Radford, S.E. Ion mobility spectrometry–mass spectrometry defines the oligomeric intermediates in amylin amyloid formation and the mode of action of inhibitors. *J. Am. Chem. Soc.* **2013**, *136*, 660–670. [CrossRef] [PubMed]
157. Meng, F.; Abedini, A.; Plesner, A.; Verchere, C.B.; Raleigh, D.P. The flavanol (−)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against IAPP-induced toxicity. *Biochemistry* **2010**, *49*, 8127–8133. [CrossRef] [PubMed]
158. Noor, H.; Cao, P.; Raleigh, D.P. Morin hydrate inhibits amyloid formation by islet amyloid polypeptide and disaggregates amyloid fibers. *Protein Sci.* **2012**, *21*, 373–382. [CrossRef] [PubMed]
159. Mishra, R.; Sellin, D.; Radovan, D.; Gohlke, A.; Winter, R. Inhibiting islet amyloid polypeptide fibril formation by the red wine compound resveratrol. *ChemBioChem* **2009**, *10*, 445–449. [CrossRef] [PubMed]
160. Radovan, D.; Opitz, N.; Winter, R. Fluorescence microscopy studies on islet amyloid polypeptide fibrillation at heterogeneous and cellular membrane interfaces and its inhibition by resveratrol. *FEBS Lett.* **2009**, *583*, 1439–1445. [CrossRef] [PubMed]
161. Jiang, P.; Li, W.; Shea, J.-E.; Mu, Y. Resveratrol inhibits the formation of multiple-layered β-sheet oligomers of the human islet amyloid polypeptide segment 22–27. *Biophys. J.* **2011**, *100*, 1550–1558. [CrossRef] [PubMed]
162. Rigacci, S.; Guidotti, V.; Bucciantini, M.; Parri, M.; Nediani, C.; Cerbai, E.; Stefani, M.; Berti, A. Oleuropein aglycon prevents cytotoxic amyloid aggregation of human amylin. *J. Nutr. Biochem.* **2010**, *21*, 726–735. [CrossRef] [PubMed]
163. Daval, M.; Bedrood, S.; Gurlo, T.; Huang, C.-J.; Costes, S.; Butler, P.C.; Langen, R. The effect of curcumin on human islet amyloid polypeptide misfolding and toxicity. *Amyloid* **2010**, *17*, 118–128. [CrossRef] [PubMed]
164. Mirhashemi, S.M.; Aarabi, M.-H. Effect of two herbal polyphenol compounds on human amylin amyloid formation and destabilization. *J. Med. Plants Res.* **2012**, *6*, 3207–3212.
165. Mirhashemi, S.M.; Aarabi, M.-H. To evaluate likely antiamyloidogenic property of ferulic acid and baicalein against human islet amyloid polypeptide aggregation, in vitro study. *Afr. J. Pharm. Pharmacol.* **2012**, *6*, 671–676.
166. Cheng, B.; Gong, H.; Li, X.; Sun, Y.; Chen, H.; Zhang, X.; Wu, Q.; Zheng, L.; Huang, K. Salvianolic acid B inhibits the amyloid formation of human islet amyloid polypeptideand protects pancreatic beta-cells against cytotoxicity. *Proteins* **2013**, *81*, 613–621. [CrossRef] [PubMed]
167. Cheng, B.; Gong, H.; Li, X.; Sun, Y.; Zhang, X.; Chen, H.; Liu, X.; Zheng, L.; Huang, K. Silibinin inhibits the toxic aggregation of human islet amyloid polypeptide. *Biochem. Biophys. Res. Commun.* **2012**, *419*, 495–499. [CrossRef] [PubMed]
168. Zelus, C.; Fox, A.; Calciano, A.; Faridian, B.S.; Nogaj, L.A.; Moffet, D.A. Myricetin inhibits islet amyloid polypeptide (IAPP) aggregation and rescues living mammalian cells from IAPP toxicity. *Open Biochem. J.* **2012**, *6*, 66–70. [CrossRef] [PubMed]

169. Aarabi, M.-H.; Mirhashemi, S.M. The role of two natural flavonoids on human amylin aggregation. *Afr. J. Pharm. Pharmacol.* **2012**, *6*, 2374–2379. [CrossRef]
170. Kao, P.-Y.; Green, E.; Pereira, C.; Ekimura, S.; Juarez, D.; Whyte, T.; Arhar, T.; Malaspina, B.; Nogaj, L.A.; Moffet, D.A. Inhibition of toxic IAPP amyloid by extracts of common fruits. *J. Funct. Foods* **2015**, *12*, 450–457. [CrossRef] [PubMed]
171. Bohn, T. Dietary factors affecting polyphenol bioavailability. *Nutr. Rev.* **2014**, *72*, 429–452. [CrossRef] [PubMed]
172. Van Duynhoven, J.P.M.; Vaughan, E.E.; Jacobs, D.M.; Kemperman, R.; van Velzen, E.J.J.; Gross, G.; Roger, L.C.; Possemiers, S.; Smilde, A.K.; Doré, J.; et al. Metabolic fate of polyphenols in the human superorganism. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4531–4538. [CrossRef] [PubMed]
173. Guo, Y.; Bruno, R.S. Endogenous and exogenous mediators of quercetin bioavailability. *J. Nutr. Biochem.* **2015**, *26*, 201–210. [CrossRef] [PubMed]
174. Sequeira, I.R.; Kruger, M.C.; Hurst, R.D.; Lentle, R.G. Ascorbic acid may exacerbate aspirin-induced increase in intestinal permeability. *Basic Clin. Pharmacol. Toxicol.* **2015**, *117*, 195–203. [CrossRef] [PubMed]
175. Gee, J.M.; DuPont, M.S.; Rhodes, M.J.; Johnson, I.T. Quercetin glucosides interact with the intestinal glucose transport pathway. *Free Radic. Biol. Med.* **1998**, *25*, 19–25. [CrossRef]
176. Day, A.J.; Cañada, F.J.; Diaz, J.C.; Kroon, P.A.; Mclauchlan, R.; Faulds, C.B.; Plumb, G.W.; Morgan, M.R.; Williamson, G. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **2000**, *468*, 166–170. [CrossRef]
177. Gee, J.M.; DuPont, M.S.; Day, A.J.; Plumb, G.W.; Williamson, G.; Johnson, I.T. Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J. Nutr.* **2000**, *130*, 2765–2771. [PubMed]
178. Day, A.J.; DuPont, M.S.; Ridley, S.; Rhodes, M.; Rhodes, M.J.; Morgan, M.R.; Williamson, G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity. *FEBS Lett.* **1998**, *436*, 71–75. [CrossRef]
179. Selma, M.V.; Espín, J.C.; Tomás-Barberán, F.A. Interaction between phenolics and gut microbiota: Role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485–6501. [CrossRef] [PubMed]
180. Moco, S.; Martin, F.-P.J.; Rezzi, S. Metabolomics view on gut microbiome modulation by polyphenol-rich foods. *J. Proteome Res.* **2012**, *11*, 4781–4790. [CrossRef] [PubMed]
181. Hollman, P.C.; de Vries, J.H.; van Leeuwen, S.D.; Mengelers, M.J.; Katan, M.B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276–1282. [PubMed]
182. Hollman, P.C.; Gaag, M.V.; Mengelers, M.J.; Van Trijp, J.M.; De Vries, J.H.; Katan, M.B. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radic. Biol. Med.* **1996**, *21*, 703–707. [CrossRef]
183. Hollman, P.C.H.; Bijsman, M.N.C.P.; van Gameren, Y.; Cnossen, E.P.J.; de Vries, J.H.M.; Katan, M.B. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radic. Res.* **1999**, *31*, 569–573. [CrossRef] [PubMed]
184. Hollman, P.C.H. The 7th international conference on polyphenols and health. *Nutr. Bull.* **2016**, *41*, 92–95. [CrossRef]
185. Galleano, M.; Verstraeten, S.V.; Oteiza, P.I.; Fraga, C.G. Antioxidant actions of flavonoids: Thermodynamic and kinetic analysis. *Arch. Biochem. Biophys.* **2010**, *501*, 23–30. [CrossRef] [PubMed]
186. Azuma, K.; Ippoushi, K.; Ito, H.; Higashio, H.; Terao, J. Combination of lipids and emulsifiers enhances the absorption of orally administered quercetin in rats. *J. Agric. Food Chem.* **2002**, *50*, 1706–1712. [CrossRef] [PubMed]
187. Guo, Y.; Mah, E.; Davis, C.G.; Jalili, T.; Ferruzzi, M.G.; Chun, O.K.; Bruno, R.S. Dietary fat increases quercetin bioavailability in overweight adults. *Mol. Nutr. Food Res.* **2013**, *57*, 896–905. [CrossRef] [PubMed]
188. Sharma, S.; Ali, A.; Ali, J.; Sahni, J.K.; Baboota, S. Rutin: Therapeutic potential and recent advances in drug delivery. *Expert Opin. Investig. Drugs* **2013**, *22*, 1063–1079. [CrossRef] [PubMed]
189. Boyle, S.; Dobson, V.; Duthie, S.; Hinselwood, D.; Kyle, J.; Collins, A. Bioavailability and efficiency of rutin as an antioxidant: A human supplementation study. *Eur. J. Clin. Nutr.* **2000**, *54*, 774–782. [CrossRef] [PubMed]
190. Wong, W.P.; Scott, D.W.; Chuang, C.-L.; Zhang, S.; Liu, H.; Ferreira, A.; Saafi, E.L.; Choong, Y.S.; Cooper, G.J. Spontaneous diabetes in hemizygous human amylin transgenic mice that developed neither islet amyloid nor peripheral insulin resistance. *Diabetes* **2008**, *57*, 2737–2744. [CrossRef] [PubMed]

191. Graefe, E.U.; Wittig, J.; Mueller, S.; Riethling, A.K.; Uehleke, B.; Drewelow, B.; Pforte, H.; Jacobasch, G.; Derendorf, H.; Veit, M. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J. Clin. Pharmacol.* **2001**, *41*, 492–499. [CrossRef] [PubMed]
192. Jaganath, I.B.; Jaganath, I.B.; Mullen, W.; Edwards, C.A.; Crozier, A. The relative contribution of the small and large intestine to the absorption and metabolism of rutin in man. *Free Radic. Res.* **2006**, *40*, 1035–1046. [CrossRef] [PubMed]
193. Chen, I.L.; Tsai, Y.-J.; Huang, C.-M.; Tsai, T.-H. Lymphatic absorption of quercetin and rutin in rat and their pharmacokinetics in systemic plasma. *J. Agric. Food Chem.* **2010**, *58*, 546–551. [CrossRef] [PubMed]
194. Andlauer, W.; Stumpf, C.; Fürst, P. Intestinal absorption of rutin in free and conjugated forms. *Biochem. Pharmacol.* **2001**, *62*, 369–374. [CrossRef]
195. Thompson, M.; Cohn, L.; Jordan, R. Use of rutin for medical management of idiopathic chylothorax in four cats. *J. Am. Vet. Med. Assoc.* **1999**, *215*, 345–348. [PubMed]
196. Zimmet, P.Z.; Magliano, D.J.; Herman, W.H.; Shaw, J.E. Diabetes: A 21st century challenge. *Lancet Diabetes Endocrinol.* **2014**, *2*, 56–64. [CrossRef]
197. Zhang, H.; Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr. Opin. Food Sci.* **2016**, *8*, 33–42. [CrossRef]
198. Atanasov, A.G.; Dzyakanchuk, A.A.; Schweizer, R.A.; Nashev, L.G.; Maurer, E.M.; Odermatt, A. Coffee inhibits the reactivation of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1: A glucocorticoid connection in the anti-diabetic action of coffee? *FEBS Lett.* **2006**, *580*, 4081–4085. [CrossRef] [PubMed]
199. Johar, H. Association of salivary cortisol levels and type 2 diabetes in the Kora-age study. *J. Psychosom. Res.* **2015**, *78*, 604. [CrossRef]
200. Chiodini, I.; Adda, G.; Scillitani, A.; Coletti, F.; Morelli, V.; Di Lembo, S.; Epaminonda, P.; Masserini, B.; Beck-Peccoz, P.; Orsi, E.; et al. Cortisol secretion in patients with type 2 diabetes. *Diabetes Care* **2007**, *30*, 83–88. [CrossRef] [PubMed]
201. Di Dalmazi, G.; Vicennati, V.; Rinaldi, E.; Morselli-Labate, A.M.; Giampalma, E.; Mosconi, C.; Pagotto, U.; Pasquali, R. Progressively increased patterns of subclinical cortisol hypersecretion in adrenal incidentalomas differently predict major metabolic and cardiovascular outcomes: A large cross-sectional study. *Eur. J. Endocrinol.* **2012**, *166*, 669–677. [CrossRef] [PubMed]
202. Hackett, R.A.; Steptoe, A.; Kumari, M. Association of diurnal patterns in salivary cortisol with type 2 diabetes in the Whitehall II study. *J. Clin. Endocrinol. Metab.* **2014**, *99*, 4625–4631. [CrossRef] [PubMed]
203. Habtemariam, S.; Lentini, G. The therapeutic potential of rutin for diabetes: An update. *Mini Rev. Med. Chem.* **2015**, *15*, 524–528. [CrossRef] [PubMed]
204. Kim, J.-H.; Kang, M.-J.; Choi, H.-N.; Jeong, S.-M.; Lee, Y.-M.; Kim, J.-I. Quercetin attenuates fasting and postprandial hyperglycemia in animal models of diabetes mellitus. *Nutr. Res. Pract.* **2011**, *5*, 107–111. [CrossRef] [PubMed]
205. Dhanya, R.; Arun, K.B.; Syama, H.P.; Nisha, P.; Sundaresan, A.; Santhosh Kumar, T.R.; Jayamurthy, P. Rutin and quercetin enhance glucose uptake in l6 myotubes under oxidative stress induced by tertiary butyl hydrogen peroxide. *Food Chem.* **2014**, *158*, 546–554. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Identification of Urinary Polyphenol Metabolite Patterns Associated with Polyphenol-Rich Food Intake in Adults from Four European Countries

Hwayoung Noh [1], Heinz Freisling [1,*], Nada Assi [1], Raul Zamora-Ros [1,2], David Achaintre [1], Aurélie Affret [3,4], Francesca Mancini [3,4], Marie-Christine Boutron-Ruault [3,4], Anna Flögel [5], Heiner Boeing [5], Tilman Kühn [6], Ruth Schübel [6], Antonia Trichopoulou [7,8], Androniki Naska [7,8], Maria Kritikou [7], Domenico Palli [9], Valeria Pala [10], Rosario Tumino [11], Fulvio Ricceri [12,13], Maria Santucci de Magistris [14], Amanda Cross [15], Nadia Slimani [1], Augustin Scalbert [1] and Pietro Ferrari [1]

1. Section of Nutrition and Metabolism, International Agency for Research on Cancer (IARC), 69372 Lyon CEDEX 08, France; NohH@fellows.iarc.fr (H.N.); AssiN@students.iarc.fr (N.A.); RaulZamoraros@gmail.com (R.Z.-R.); AchaintreD@iarc.fr (D.A.); SlimaniN@iarc.fr (N.S.); ScalbertA@iarc.fr (A.S.); FerrariP@iarc.fr (P.F.)
2. Unit of Nutrition and Cancer, Epidemiology Research Program, Catalan Institute of Oncology, Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet de Llobregat, 08908 Barcelona, Spain
3. Université Paris-Saclay, Université Paris-Sud, Université de Versailles Saint-Quentin-en-Yvelines (UVSQ), Le Centre de recherche en Epidémiologie et Santé des Population (CESP), Institut National de la Santé et de la Recherche Médicale (INSERM), 94800 Villejuif, France; Aurelie.AFFRET@gustaveroussy.fr (A.A.); Francesca.Mancini@gustaveroussy.fr (F.M.); Marie-christine.BOUTRON@gustaveroussy.fr (M.-C.B.-R.)
4. Gustave Roussy, 94800 Villejuif, France
5. Department of Epidemiology, German Institute of Human Nutrition Potsdam-Rehbrücke, 14558 Nuthetal, Germany; Anna.Floegel@dife.de (A.F.); boeing@dife.de (H.B.)
6. Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany; T.Kuehn@Dkfz-Heidelberg.de (T.K.); R.Schuebel@dkfz-heidelberg.de (R.S.)
7. Hellenic Health Foundation, 115 27 Athens, Greece; atrichopoulou@hhf-greece.gr (A.T.); anaska@hhf-greece.gr (A.N.); maria.kritikou@hhf-greece.gr (M.K.)
8. WHO Collaborating Center for Nutrition and Health, Unit of Nutritional Epidemiology and Nutrition in Public Health, Department of Hygiene, Epidemiology and Medical Statistics, University of Athens Medical School, 157 72 Athens, Greece
9. Cancer Risk Factors and Life-Style Epidemiology Unit, Cancer Research and Prevention Institute (ISPO), 50139 Florence, Italy; d.palli@ispo.toscana.it
10. Epidemiology and Prevention Unit, Department of Preventive and Predictive Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milano, Italy; Valeria.Pala@istitutotumori.mi.it
11. Cancer Registry and Histopathology Unit, "Civic-M.P.Arezzo" Hospital, ASP Ragusa, 97100 Ragusa, Italy; rosario.tumino@asp.rg.it
12. Unit of Epidemiology, Regional Health Service ASL TO3, 10095 Grugliasco (TO), Italy; fulvio.ricceri@unito.it
13. Unit of Cancer Epidemiology, Department of Medical Sciences, University of Turin, 10124 Turin, Italy
14. Azienda Ospedaliera Universitaria (AOU) Federico II, 80131 Naples, Italy; masantuc@unina.it
15. Department of Epidemiology and Biostatistics, School of Public Health, Imperial College London, St Mary's Campus, Norfolk Place, London W2 1PG, UK; amanda.cross@imperial.ac.uk

* Correspondence: FreislingH@iarc.fr; Tel.: +33-4-7273-8664

Received: 30 June 2017; Accepted: 18 July 2017; Published: 25 July 2017

Abstract: We identified urinary polyphenol metabolite patterns by a novel algorithm that combines dimension reduction and variable selection methods to explain polyphenol-rich food intake, and compared their respective performance with that of single biomarkers in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The study included 475 adults from four European countries (Germany, France, Italy, and Greece). Dietary intakes were assessed with 24-h dietary recalls (24-HDR) and dietary questionnaires (DQ). Thirty-four polyphenols were measured

by ultra-performance liquid chromatography–electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS-MS) in 24-h urine. Reduced rank regression-based variable importance in projection (RRR-VIP) and least absolute shrinkage and selection operator (LASSO) methods were used to select polyphenol metabolites. Reduced rank regression (RRR) was then used to identify patterns in these metabolites, maximizing the explained variability in intake of pre-selected polyphenol-rich foods. The performance of RRR models was evaluated using internal cross-validation to control for over-optimistic findings from over-fitting. High performance was observed for explaining recent intake (24-HDR) of red wine (r = 0.65; AUC = 89.1%), coffee (r = 0.51; AUC = 89.1%), and olives (r = 0.35; AUC = 82.2%). These metabolite patterns performed better or equally well compared to single polyphenol biomarkers. Neither metabolite patterns nor single biomarkers performed well in explaining habitual intake (as reported in the DQ) of polyphenol-rich foods. This proposed strategy of biomarker pattern identification has the potential of expanding the currently still limited list of available dietary intake biomarkers.

Keywords: dietary biomarker patterns; polyphenol metabolites; polyphenol-rich food; reduced rank regression (RRR); EPIC

---

1. Introduction

In nutritional epidemiology, the accurate and precise estimation of dietary exposures is critical for an unbiased assessment of diet–disease associations. Intakes of foods, nutrients or other bioactive compounds related to health or diseases are often estimated using self-reported dietary assessment methods, such as 24-h dietary recalls (24-HDR) or dietary questionnaires (DQs). However, the reliability of traditional self-reported instruments has been challenged due to inherent and sizeable measurement errors [1,2]. Dietary measurement errors are a serious challenge to establish reliable diet–disease associations [3].

Over the last decades, a limited number of dietary biomarkers have been identified and implemented in nutritional epidemiology [4,5]. They have been useful as reference measurements to validate self-reported dietary assessment tools (i.e., doubly labeled water and urinary nitrogen), as complementary measurements to compare with estimates of dietary intake (i.e., fatty acids, and carotenoids in blood), or as substitute measurements for insufficient or unavailable dietary intake data (i.e., selenium and zinc in blood) [4,6]. More recently, with the development of metabolomics, novel dietary biomarkers are being identified that should further improve the accuracy of dietary intake estimation [7,8].

These biomarkers have been mostly used individually for dietary exposure assessment. However, the 'single biomarker' approach has some conceptual and methodological limitations. First, single biomarkers cannot reflect complex matrices of dietary exposures with various food groups, which consist of multiple nutrients and other food components converted to a number of metabolites through various biological pathways, including the gut microbiota. Also, there are high inter-correlations among biomarkers, and some biomarker levels are too low to be detected or to reach a statistically significant performance for use in dietary intake assessment. Therefore, a 'biomarker pattern' approach may provide a more comprehensive and accurate measurement of complex dietary exposures. In this respect, some recent studies have used combinations of dietary biomarkers to improve the accuracy of dietary exposures assessment [9,10].

Polyphenols are non-nutritive plant components widely distributed in a variety of foods including fruits, vegetables, tea, coffee and wine [11]. Research interest in polyphenols has increased due to their potential protective effects on non-communicable diseases including cardiovascular diseases, diabetes and cancer, and premature mortality [12–15]. Overall results may be promising, but they remain inconclusive, and further prospective studies assessing dietary polyphenol exposure and

studies using other methods to evaluate exposure (i.e., markers of consumption, metabolism, excretion) have been recommended, as concluded in a recent meta-analysis summarizing available evidence on the association of dietary flavonoid and lignan intake with cancer risk in observational studies [15]. Polyphenol metabolites measured in biological specimens could complement traditional dietary assessment tools to improve exposure assessment. A recent systematic review using intervention studies confirmed that urinary polyphenol metabolites could serve as dietary biomarkers with high recovery yields and high correlations with intakes of polyphenol-rich food [16]. Some single urinary polyphenol metabolites, such as, for example, chlorogenic acid/caffeic acid, gallic acid/resveratrol, caffeic acid/epicatechin, and naringenin/hesperetin have been identified as potential biomarkers for intakes of coffee, wine, tea, and citrus fruits/juices, respectively [17–20]. However, we hypothesized that panels or patterns of polyphenol metabolites may better explain intake of polyphenol-containing foods.

Recently, we reported correlations of 34 individual urinary polyphenol metabolites with intake of polyphenol-containing foods in the European Prospective Investigation into Cancer and Nutrition (EPIC) cross-sectional study [20]. Some single polyphenols were found to be significantly correlated to recent intake of these foods and were proposed as potential biomarkers of intake for these foods. In the current study, the same data were used to identify patterns of urinary polyphenol metabolites by applying a new algorithm that combines dimension reduction and variable selection methods to maximize the explained variation in intake of specific polyphenol-rich foods. The ability of these urinary polyphenol patterns to rank individuals according to the intake and to discriminate between consumers and non-consumers was examined and compared with the respective performance of single polyphenols.

2. Materials and Methods

2.1. Subjects

This study included 475 subjects randomly selected from four European countries (i.e., Germany, France, Italy, and Greece) within the EPIC calibration study, as described in our previous study [20]. In brief, the EPIC study is an ongoing multi-center prospective cohort study with more than half a million subjects, mostly aged 35–70 years, recruited from 23 centers in 10 European countries between 1992–2000. The study was designed to investigate relations between diet, lifestyle and environmental factors, and the risk of cancer and other chronic disease by collecting information on diet and lifestyle characteristics, anthropometric measurements, and medical history [21]. For the EPIC calibration study, a single 24-HDR was collected from a random sub-sample (n = 36,900) of the entire cohort, and a 24-h urine specimen was collected from a convenient sub-sample between 1995–1999 (n = 1386) of the calibration study [22,23]. For the current study, all subjects with available data in the form of a 24-h urine specimen and a 24-HDR collected on the same day, and a country-specific validated DQ collected at different time intervals (1 day–34 months) with regard to the 24-h urine collection across centers (n = 475), were eligible [24]. These subjects were recruited from the general population residing within defined geographical areas in Germany (Heidelberg and Potsdam), Greece (nationwide) and Italy (Naples, Turin, and Varese). Subjects had come to the study from breast cancer screening in Florence, a local blood donors association and their partners in Ragusa, Italy, and from an existing cohort. The latter was the case in France, where there was a cohort based on female teachers and school workers (Paris and surrounding areas). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethical review boards of the International Agency for Research on Cancer (IARC) and from local participating institutions (Project identification code: doc. SC/24/6, date of approval: September 1987).

2.2. Dietary Assessment

Dietary data were collected using a single standardized 24-HDR and a country-specific validated DQ. The 24-HDR face-to-face interview was conducted using a standardized dietary assessment methodology with a computerized program (EPIC-Soft) [22,25]. Dietary intake data using DQ with 158~266 items were self-administered or collected by face-to-face interviews to estimate usual intake over the previous 12 months [22].

2.3. Urinary Polyphenol Assessment

24-h urines were used for the measurement of urinary polyphenols. For the collection, subjects were provided two 2-L containers, each with 2 g boric acid as preservative. P-Aminobenzoic acid (PABA) was used as a marker for completeness of 24-h urine collections. After collection, 24-h urine samples were stored at −20 °C at the local center, and finally shipped within 24 h to and stored at −20 °C at the IARC, where laboratory analyses were performed after about 15 years of storage [19]. We do not expect major degradation of polyphenol metabolites during storage, and our previous studies using the same urine samples showed expected correlations between the metabolites and food intake [19,20]. As described previously [26], urine samples were first hydrolyzed with a β-glucuronidase/sulfatase enzyme mixture and the resulting polyphenol aglycones were extracted twice with ethyl acetate. Quantitative dansylation of phenolic hydroxyl groups was carried out with either 13C-dansyl chloride (samples) or non-labeled 12C-dansyl chloride (well-characterized reference pooled sample). Each 13C-dansylated sample was mixed with the 12C-dansylated reference sample, and the relative concentrations in samples over the reference were then measured by ultra-performance liquid chromatography–electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). A total of 37 urinary polyphenols were measured, and their excretions in urine were expressed as µmol/24-h. Urinary polyphenol concentrations below the limit of quantification (LOQ) were replaced with values for half the LOQ. Since 98–100% of three polyphenols (procyanidins B1 and B2, and (+)-gallocatechin) values were below the LOQ, they were excluded from the analysis.

2.4. Statistical Analyses

Prior to the main statistical analyses, missing values of polyphenols were imputed by the expectation-maximization (EM) algorithm [27] after log transformation. In our previous study, center and batch were shown to explain a large part of the total variability of urinary polyphenols [20]. Urinary polyphenol measurements were therefore adjusted by taking the residuals from general linear models (GLMs), with center and batch variables as covariates. Intakes of food groups were log transformed and adjusted for energy intake by taking the residuals from GLMs with energy intake variable. Partial Pearson's correlations between 34 individual polyphenols and the intakes of 12 main food groups and their 144 sub-groups (see Tables S1–S5) were computed conditional on sex, body mass index (BMI) and age as covariates.

An algorithm using dimension reduction and variable selection methods were applied to identify patterns of polyphenol metabolites and best explain the intake of polyphenol-rich foods. The procedure of the algorithm was as follows:

(1) Selecting optimal subsets of 34 polyphenol metabolites to explain intakes of specific polyphenol-rich food groups using two different variable selection methods: (i) variable importance in projection based on reduced rank regression (called the RRR-VIP method) [28] and (ii) least absolute shrinkage and selection operator (LASSO) regression [29].
(2) Identifying patterns of selected polyphenol metabolites (as predictor variables), and maximizing the explained variability of polyphenol-rich food group intakes (as response variables) through RRR analysis.
(3) Evaluating the performance of the RRR models for the polyphenol metabolite patterns to discriminate between consumers and non-consumers through internal two-fold cross-validation

analyses. This was achieved through splitting the data into two equal-sized subsets (a training and a test set) and calculating (i) RRR scores in the test set using factor weights derived from RRR analysis of the training set; and (ii) Pearson correlation coefficients of RRR scores with intakes and area under the receiver operating characteristic curves (ROC AUCs) for the RRR scores of the test set.

For variable selection using the RRR-VIP method, a VIP score of each polyphenol metabolite, which is a weighted sum of squares of the RRR weights accounting for the explained variance of each RRR model, was calculated, and then polyphenol metabolites with a VIP score greater than 0.85 were selected [28,30]. Alternatively, we applied LASSO regression and its five-fold cross validation to select subsets of polyphenol metabolites by shrinking i.e., setting to 0, some coefficients of the predictors [29]. Partial Pearson correlation coefficients of RRR scores with intakes of polyphenol-rich foods from 24-HDR or DQ were calculated conditional on covariates (sex, BMI and age), and ROC AUCs were adjusted for these same covariates. All analyses were conducted using the Statistical Analysis Software, release 9.4 (SAS Institute Inc., Cary, NC, USA) and R software, version R.3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. General Characteristics of the Study Population

The average age and BMI of the participants were 54 ± 8.5 years and 26 ± 4.3 kg/m^2, respectively. The percentage of smokers (former/current) and never smokers was 62% and 36% in men, and 36% and 61% in women, respectively. The proportion of subjects with prevalent diabetes, hyperlipidemia or hypertension was 2.5%, 27.2% and 23.6%, respectively (Table 1).

Table 1. General characteristics [a] of the total study population (n = 475).

	Total	Men	Women	p [b]
N (%)	475 (100)	198 (41.7)	277 (58.3)	
Age (years)	53.9 (8.5)	55.4 (8.4)	52.9 (8.4)	0.017
BMI (kg/m^2)	26.0 (4.3)	26.8 (3.5)	25.5 (4.7)	0.059
Energy intake (kcal/day)	2200.0 (785.5)	2562.7 (830.9)	1940.8 (636.4)	<0.0001
Alcohol intake (g/day)	15.5 (21.1)	23.5 (26.3)	9.7 (13.8)	<0.0001
Smoking status (%)				0.102
Never	50.7	35.9	61.4	
Former	27.2	38.4	19.1	
Current	19.4	23.2	16.6	
Unknown	2.7	2.5	2.9	
Physical activity (%)				0.712
Inactive	26.3	24.8	27.4	
Moderately inactive	40.0	39.9	40.1	
Moderately active	21.3	21.2	21.3	
Active	12.4	14.1	11.2	
Diabetes (%) [c]	2.5	3.5	1.8	0.213
Hyperlipidemia (%) [c]	27.2	33.3	22.7	0.087
Hypertension (%) [c]	23.6	27.8	20.7	0.720

[a] Mean (SD) or Percentage (%); [b] p-values for the difference between men and women from the regression—center-adjusted linear regression (continuous variables) or logistic regression (categorical variables); [c] Self-reported by questionnaires at recruitment into the study.

3.2. Correlations between Individual Polyphenol Metabolites and Polyphenol-Rich Food Groups

In a first step, correlations between 34 individual polyphenol metabolites and the intakes of 12 main food groups and their 144 sub-groups in the EPIC study were explored to pre-select specific

food groups that had sufficiently high correlations with a minimum set of polyphenol metabolites. Among the main food groups investigated, only four ('vegetables', 'fruit, nuts & seeds', 'non-alcoholic beverages', and 'alcoholic beverages') were significantly correlated with more than five individual polyphenol metabolites, while other main food groups were significantly correlated with less than three individual metabolites, and all coefficients were below 0.2 (Table S1). In a subsequent step, we examined correlations between polyphenol metabolites and food sub-groups (Tables S2–S5). Among these sub-groups, citrus fruits, apples and pears, olives, coffee, tea, all wine, and red wine were highly correlated with individual polyphenols (Table 2). For example, highly-correlated polyphenols were hesperetin (r = 0.54) and naringenin (r = 0.50) for citrus fruits, caffeic acid (r = 0.49) and ferulic acid (r = 0.42) for coffee, and gallic acid ethyl ester (r = 0.65) and resveratrol (r = 0.46) for red wine. All these food groups were selected for our multivariate analyses as polyphenol-rich food groups. The a priori arbitrarily defined criteria were that a given food group (or sub-group) showed a significant correlation with at least five polyphenols, and that at least one of these correlations was $r \geq 0.3$. The criteria were chosen as a trade-off between having sufficiently informative predictor variables (i.e., polyphenols) and a wider range of potential food groups (i.e., response variables).

Table 2. Correlation coefficients [a] between urinary polyphenols and intakes of polyphenol-rich foods from 24-HDR among total subjects (n = 475).

Polyphenols (n = 34)	Food Groups (% Consumers)						
	Citrus Fruits (38.9%)	Apple & Pear (47.6%)	Olives (9.3%)	Coffee (86.3%)	Tea (24.6%)	All Wine (41.9%)	Red Wine (25.5%)
Protocatechuic acid	0.020	0.018	0.055	0.373	−0.116	0.119	0.109
Hydroxytyrosol	0.020	0.010	0.360	0.010	0.100	0.430	0.336
3,5-Dihydroxybenzoic acid	0.080	0.023	0.034	−0.093	0.130	−0.016	−0.027
3,4-Dihydroxyphenylacetic acid	0.174	0.134	0.312	0.028	0.053	0.134	0.116
Genistein	0.076	0.018	−0.027	−0.093	0.067	−0.072	−0.047
Apigenin	0.088	0.055	0.014	−0.062	−0.027	−0.081	−0.064
3,4-Dihydroxyphenylpropionic acid	0.062	0.086	0.012	0.403	−0.159	0.038	0.025
3,5-Dihydroxyphenylpropionic acid	0.077	0.022	0.020	−0.043	0.142	0.050	0.055
3-Hydroxybenzoic acid	0.029	0.024	−0.013	0.162	0.077	0.052	0.091
4-Hydroxybenzoic acid	0.191	−0.031	0.071	0.094	0.008	0.009	0.010
Tyrosol	−0.079	−0.084	0.117	0.045	0.037	0.429	0.317
3-Hydroxyphenylacetic acid	0.121	0.141	0.058	0.027	0.034	0.060	0.063
4-Hydroxyphenylacetic acid	−0.014	−0.060	0.054	0.012	−0.011	0.220	0.164
m-Coumaric acid	0.054	−0.022	0.001	0.294	−0.092	0.113	0.128
p-Coumaric acid	0.011	0.088	0.126	0.104	0.061	0.270	0.212
Vanillic acid	−0.014	0.000	0.009	0.107	−0.065	−0.017	0.024
Naringenin	0.498	0.070	0.064	0.036	−0.018	0.025	−0.043
Phloretin	0.151	0.303	−0.009	0.000	−0.005	−0.027	−0.057
Kaempferol	0.279	0.085	0.036	0.003	0.083	−0.002	−0.021
Epicatechin	0.020	0.233	−0.015	−0.126	0.193	0.135	0.123
Catechin	−0.069	0.003	0.018	−0.098	0.110	0.280	0.280
Hesperetin	0.535	0.056	0.023	0.037	−0.061	0.004	−0.003
Homovanillic acid	0.126	0.117	0.241	−0.081	0.059	0.065	0.069
Isorhamnetin	0.032	0.070	0.036	−0.055	0.074	0.047	0.078
Ferulic acid	0.170	0.053	0.028	0.422	−0.113	0.036	0.003
Resveratrol	0.028	−0.049	0.007	0.012	−0.007	0.409	0.457
Quercetin	0.190	0.083	−0.008	−0.118	0.133	0.126	0.141
Caffeic acid	0.068	0.092	0.049	0.487	−0.121	0.119	0.084
Equol	−0.060	−0.068	−0.040	−0.099	0.049	0.009	0.060
Daidzein	0.043	−0.037	−0.008	−0.115	0.089	−0.023	−0.029
Enterolactone	0.050	0.045	0.105	0.019	0.042	0.077	0.032
Enterodiol	0.067	0.000	0.053	−0.015	0.016	0.018	0.027
Gallic acid	0.055	0.064	0.039	−0.125	0.316	0.344	0.380
Gallic acid ethyl ester	−0.016	−0.030	0.032	−0.009	0.058	0.508	0.654

[a] Partial Pearson correlation with sex, BMI and age as covariates. Urinary polyphenols were adjusted for center and batch and intakes of food groups were adjusted for energy intake using residuals from general linear models (GLMs). Positive coefficients in blue cells were significant ($p < 0.05$) and higher coefficients had darker color.

3.3. Selection of Polyphenol Metabolites Using Variable Selection Methods

Out of 34 urinary polyphenol metabolites, sub-sets were selected for identifying patterns associated with intakes of polyphenol-rich food groups using the RRR-VIP method and LASSO regression. Selected polyphenols differed by method, but at least the first one or two polyphenols were common in both methods (Table 3).

Table 3. Selected polyphenol metabolites [a] by reduced rank regression-based variable importance in projection (RRR-VIP) or least absolute shrinkage and selection operator (LASSO) methods (n = 475).

Food Groups	RRR-VIP		LASSO	
	Polyphenol Metabolites	VIP	Polyphenol Metabolites	Coefficients
Citrus fruits	**Naringenin**	2.876	**Hesperetin**	0.851
	Hesperetin	2.701	**Naringenin**	0.510
	3,4-Dihydroxyphenylacetic acid	2.552	**3,4-Dihydroxyphenylacetic acid**	0.091
	Resveratrol	1.207	3-Hydroxyphenylacetic acid	0.086
	3,4-Dihydroxyphenylpropionic acid	1.007	Vanillic acid	−0.009
	m-Coumaric acid	0.970	Apigenin	−0.035
	Genistein	0.927	Tyrosol	−0.037
	Homovanillic acid	0.894	**Catechin**	−0.046
	Catechin	0.888	4-Hydroxyphenylacetic acid	−0.089
	Daidzein	0.880		
	Hydroxytyrosol	0.855		
Apples & Pears	**Phloretin**	2.666	**Phloretin**	0.598
	Epicatechin	2.463	**Epicatechin**	0.199
	Protocatechuic acid	2.047		
	Gallic acid ethyl ester	1.426		
	3,4-Dihydroxyphenylpropionic acid	1.254		
	Enterolactone	1.163		
	Catechin	1.119		
	3,4-Dihydroxyphenylacetic acid	0.914		
	Homovanillic acid	0.913		
	Apigenin	0.889		
	Daidzein	0.883		
Olives	**Hydroxytyrosol**	4.866	**Hydroxytyrosol**	0.313
	Tyrosol	1.810	**3,4-Dihydroxyphenylacetic acid**	0.099
	Quercetin	1.382	Catechin	−0.003
	3,4-Dihydroxyphenylacetic acid	0.945	m-Coumaric acid	−0.006
	Gallic acid ethyl ester	0.863	Epicatechin	−0.011
			3-Hydroxybenzoic acid	−0.014
			Gallic acid ethyl ester	−0.028
			Resveratrol	−0.041
			Tyrosol	−0.054
			Quercetin	−0.078
Coffee	**Caffeic acid**	3.559	**Caffeic acid**	0.853
	Ferulic acid	1.906	**Ferulic acid**	0.227
	3,4-Dihydroxyphenylacetic acid	1.690	**Protocatechuic acid**	0.075
	Gallic acid	1.493	**3,4-Dihydroxyphenylpropionic acid**	0.071
	Apigenin	1.270	**Homovanillic acid**	−0.013
	Quercetin	1.261	Catechin	−0.016
	Homovanillic acid	1.149	3,5-Dihydroxyphenylpropionic acid	−0.027
	Protocatechuic acid	1.141	4-Hydroxyphenylacetic acid	−0.041
	m-Coumaric acid	1.037	Equol	−0.047
	Hydroxytyrosol	0.879	3,5-Dihydroxybenzoic acid	−0.069
	Daidzein	0.872	**Daidzein**	−0.076
			Epicatechin	−0.109
			Gallic acid	−0.130
			Apigenin	−0.163
			Quercetin	−0.263

Table 3. *Cont.*

Food Groups	RRR-VIP		LASSO	
	Polyphenol Metabolites	VIP	Polyphenol Metabolites	Coefficients
Tea	**Gallic acid**	3.265	**Gallic acid**	0.977
	Hydroxytyrosol	2.084	**3-Hydroxybenzoic acid**	0.328
	Protocatechuic acid	1.813	**Hydroxytyrosol**	0.255
	3,4-Dihydroxyphenylacetic acid	1.520	**3,5-Dihydroxyphenylpropionic acid**	0.233
	3-Hydroxybenzoic acid	1.462	Kaempferol	0.177
	m-Coumaric acid	1.379	Daidzein	0.140
	3,5-Dihydroxyphenylpropionic acid	0.969	4-Hydroxybenzoic acid	0.072
	Resveratrol	0.959	Genistein	0.057
	Gallic acid ethyl ester	0.857	p-Coumaric acid	0.044
			Epicatechin	0.037
			Quercetin	0.021
			Isorhamnetin	0.018
			Enterodiol	0.006
			Ferulic acid	−0.001
			Apigenin	−0.017
			Tyrosol	−0.037
			3,4-Dihydroxyphenylpropionic acid	−0.109
			3,4-Dihydroxyphenylacetic acid	−0.112
			Gallic acid ethyl ester	−0.119
			3-Hydroxyphenylacetic acid	−0.133
			Phloretin	−0.145
			Hesperetin	−0.148
			4-Hydroxyphenylacetic acid	−0.204
			m-Coumaric acid	−0.260
			Resveratrol	−0.321
			Protocatechuic acid	−0.452
All wine	**Hydroxytyrosol**	3.547	**Gallic acid ethyl ester**	0.808
	Gallic acid ethyl ester	3.058	**Hydroxytyrosol**	0.579
	Homovanillic acid	1.531	Tyrosol	0.198
	3-Hydroxybenzoic acid	1.201	Gallic acid	0.068
	Naringenin	1.081	p-Coumaric acid	0.060
	3,4-Dihydroxyphenylpropionic acid	1.010	Enterolactone	0.048
	3,4-Dihydroxyphenylacetic acid	0.909	Catechin	0.023
			Apigenin	−0.063
			3-Hydroxybenzoic acid	−0.089
			Vanillic acid	−0.097
			Homovanillic acid	−0.251
Red wine	**Gallic acid ethyl ester**	5.388	**Gallic acid ethyl ester**	1.333
	Resveratrol	1.315		

[a] Polyphenols in bold were selected by both RRR-VIP and LASSO methods. The positive (blue) or negative (red) association of selected polyphenols with intakes of food/food groups were shown in different colors.

3.4. Identification of Polyphenol Metabolite Patterns Using Reduced Rank Regression

Patterns of selected polyphenol metabolites were identified through RRR analyses. Correlation coefficients and the AUC for the RRR scores of the polyphenol metabolite patterns were examined in the test set for cross-validation (Table 4). The RRR scores were highly correlated, with recent intakes of red wine ($r_{RRR\text{-}VIP}$ = 0.65; r_{LASSO} = 0.66), citrus fruit ($r_{RRR\text{-}VIP}$ = 0.54; r_{LASSO} = 0.54), and coffee ($r_{RRR\text{-}VIP}$ = 0.51; r_{LASSO} = 0.51) as estimated from 24-HDR (Table 4). According to the AUC for recent intakes assessed from 24-HDR, the best discrimination between consumers and non-consumers for RRR scores of polyphenol patterns was observed for coffee ($AUC_{RRR\text{-}VIP}$ = 89.1%, 95% CI = 82.9–95.4%; AUC_{LASSO} = 89.6%, 95% CI = 83.6–95.6%), followed by red wine ($AUC_{RRR\text{-}VIP}$ = 89.1%, 95% CI = 83.5–94.7%; AUC_{LASSO} = 89.1%, 95% CI = 83.6–94.7%), olives ($AUC_{RRR\text{-}VIP}$ = 82.2%, 95% CI = 72.9–91.6%; AUC_{LASSO} = 81.0%, 95% CI = 70.9–91.2%), and citrus fruits ($AUC_{RRR\text{-}VIP}$ = 81.7%, 95% CI = 76.2–87.2%; AUC_{LASSO} = 81.8%, 95% CI = 76.1–87.5%). When compared with those for single polyphenols, the performance has been improved using polyphenol patterns identified though RRR, especially for coffee (r = 0.51, AUC = 89.1% for PPs pattern vs. r = 0.42, AUC = 85.8% for single PP) and olives (r = 0.35, AUC = 82.2% for PPs pattern vs. r = 0.29, AUC = 79.6% for single PP). Correlation

coefficients and AUCs for 24-HDR data were consistently higher than those for DQ data. AUCs for habitual dietary intake assessed with DQ were all lower than 80%, except for intake of coffee ($r_{\text{RRR-VIP}}$ = 0.39; $\text{AUC}_{\text{RRR-VIP}}$ = 82.7%, 95% CI = 72.2–93.2% and r_{LASSO} = 0.42; $\text{AUC}_{\text{LASSO}}$ = 83.4%, 95% CI = 73.0–93.7% for PPs pattern vs. r = 0.38; AUC = 80.9%, 95% CI = 68.9–92.8% for single PP) (Table 4). Table S6 shows the correlation coefficients and AUC for the RRR scores of the polyphenol metabolite patterns in all subjects without applying a cross-validation. These results represent thus a potential upper limit of performance in explaining polyphenol-rich food intake, but cannot be generalized to an independent dataset.

Table 4. Correlations coefficients and area under the receiver operating characteristic curves (ROC AUCs) of RRR scores of selected polyphenol (PP) metabolites with polyphenol-rich foods from 24-HDR and DQ in the test set (n = 236).

Food Groups [a]	Selected PPs [b]	24-HDR			DQ		
		Consumers (%)	r [c]	ROC AUC [d] (95% CI)	Consumers (%)	r [c]	ROC AUC [d] (95% CI)
Citrus fruit	Single PP (Hesperetin)	40%	0.538	81.4% (75.9–86.8)	96%	0.124	66.2% (48.8–83.6)
	PPs by RRR-VIP (n = 11)		0.543	81.7% (76.2–87.2)		0.139	71.6% (57.9–85.2)
	PPs by LASSO (n = 11)		0.539	81.8% (76.1–87.5)		0.163	69.8% (54.9–84.7)
Apples & Pears	Single PP (Phloretin)	48%	0.322	74.2% (68.0–80.5)	96%	0.183	70.6% (54.0–87.2)
	PPs by RRR-VIP (n = 10)		0.359	73.5% (67.2–79.8)		0.242	77.7% (61.5–93.9)
	PPs by LASSO (n = 2)		0.356	74.3% (68.0–80.6)		0.201	68.5% (51.7–85.3)
Olives	Single PP (Hydroxytyrosol)	8%	0.287	79.6% (69.7–89.5)	26%	0.141	64.8% (56.7–72.9)
	PPs by RRR-VIP (n = 5)		0.351	82.2% (72.9–91.6)		0.131	64.1% (55.8–72.4)
	PPs by LASSO (n = 10)		0.348	81.0% (70.9–91.2)		0.125	64.2% (56.0–72.5)
Coffee	Single PP (Caffeic acid)	86%	0.416	85.8% (77.7–93.8)	94%	0.383	80.9% (68.9–92.8)
	PPs by RRR-VIP (n = 11)		0.505	89.1% (82.9–95.4)		0.392	82.7% (72.2–93.2)
	PPs by LASSO (n = 15)		0.510	89.6% (83.6–95.6)		0.417	83.4% (73.0–93.7)
Tea	Single PP (Gallic acid)	25%	0.304	70.5% (62.8–78.2)	64%	0.151	59.8% (52.2–67.5)
	PPs by RRR-VIP (n = 9)		0.412	73.9% (66.4–81.4)		0.289	65.0% (57.9–72.1)
	PPs by LASSO (n = 26)		0.370	72.4% (65.0–79.8)		0.210	63.2% (55.9–70.5)
All wine	Single PP (Gallic acid ethyl ester)	37%	0.514	76.7% (70.1–83.4)	85%	0.406	74.8% (66.4–83.2)
	PPs by RRR-VIP (n = 7)		0.529	77.8% (71.3–84.4)		0.423	76.1% (68.1–84.1)
	PPs by LASSO (n = 11)		0.531	77.1% (70.8–83.4)		0.433	76.7% (68.4–84.9)
Red Wine	Single PP (Gallic acid ethyl ester)	23%	0.656	89.1% (83.6–94.7)	24%	0.263	67.8% (59.1–76.4)
	PPs by RRR-VIP (n = 2)		0.654	89.1% (83.5–94.7)		0.263	67.8% (59.1–76.4)
	PPs by LASSO (n = 1)		0.656	89.1% (83.6–94.7)		0.263	67.8% (59.1–76.4)

[a] Intakes of food groups were adjusted for energy intake using residuals from general linear models (GLMs); [b] Polyphenol metabolites were adjusted for centers and batches using residuals from GLMs; [c] Partial Pearson correlation coefficients between RRR scores of selected polyphenols and food groups with sex, BMI and age as covariates; [d] ROC AUCs for RRR scores of the patterns of selected polyphenols were calculated and adjusted for sex, BMI and age using logistic regression models.

4. Discussion

We developed a novel statistical algorithm using a combination of dimension reduction and variable selection methods to integrate high-dimensional biomarker data, with the goal to complement self-reported dietary assessment methods and to improve dietary intake estimation in nutritional epidemiological studies. Here, we applied this approach to a panel of polyphenol metabolites measured in human urine and related dietary intake data. Among 34 targeted urinary polyphenol metabolites, optimal sub-sets were selected by RRR-VIP and LASSO methods, and these patterns of polyphenol metabolites derived by RRR models outperformed any single best polyphenol metabolite associated with the intake of polyphenol-rich foods, especially for coffee and olives.

Polyphenols are widely distributed in plant-based foods such as fruits, vegetables, tea, coffee and wine [11]. A previous study on dietary polyphenol intake in European countries [31] reported an average intake range of total polyphenol of 744–1786 mg/day and 584–1626 mg/day in men and women, respectively, and the main food sources of polyphenols were coffee (21–36%), tea (17–41%),

fruits (9–25%), wine (10%) in Mediterranean (MED) countries, non-MED countries and the UK. In this study, polyphenol-rich food groups were pre-selected based on the correlation between food groups and individual polyphenol metabolites prior to the main analyses. Similar to the previous study, fruits (citrus fruits, apples and pears, and olives), coffee, tea, and wine food groups were also selected as polyphenol-rich food groups. Despite vegetables being regarded as a food group rich in polyphenols generally, none of the vegetable sub-groups reached our criteria for being selected as a polyphenol-rich food group. This might be explained by the observation that vegetables overall contributed only less than 5% to polyphenol intake in the EPIC study [31], and different vegetable sub-groups may thus contribute only marginally to polyphenol intake, at least in the EPIC populations.

Recently, individual polyphenol metabolites have been identified as potential biomarkers of dietary polyphenol intake [32,33]. A number of studies examined the potential role of polyphenols as dietary biomarkers in clinical trials or observational studies [17,18,34–40]. Previous dietary intervention studies [34–37] have identified that flavonoids such as hesperetin, naringenin, kaempferol, phloretin, and quercetin in 24-h urine could be specific biomarkers for intakes of fruits and vegetables. Other clinical and observational studies [17,38–40] found that some 24-h urinary polyphenols were good indicators of polyphenol-rich beverage consumption, such as gallic acids and resveratrol for wine, chlorogenic acid for coffee, and epicatechin for tea. However, all these previous studies examined individual polyphenol metabolites, and to the best of our knowledge, this is the first study using polyphenol metabolite patterns to investigate associations with food intake.

Conceptually similar to dietary pattern analyses [41], free-living people do not consume single polyphenols, but a combination of polyphenols coming from different food sources. Therefore, it is meaningful from a biological point of view to examine combinations of polyphenols, which is also a more comprehensive and efficient approach from a statistical point of view. In this study, we applied dimension reduction and variable selection methods to identify specific urinary polyphenol metabolite patterns associated with the intake of polyphenol-rich foods. RRR analysis is a multivariate dimension reduction technique to determine linear combinations of a set of predictors maximizing the explained variability in responses. RRR has been previously used along with principal component analysis, factor analyses, or cluster analyses for dietary pattern discovery in nutritional epidemiology [42,43]. RRR analysis is similar to partial least squares (PLS) analysis; they are both widely used in analyses of metabolomics data, and both are supervised approaches with regression-based models to reduce dimensions by extracting linear combinations of X-variables that explain variability in Y-variables [44,45]. The difference between RRR and PLS is that RRR focuses on explaining variation in Y-variables, whereas PLS seeks factors whereby the covariance between the X- and the Y-components is maximized. Therefore, in this study, applying the RRR method enabled the identification of patterns of polyphenol metabolites that maximized the explained variability of intakes of specific polyphenol-rich food groups.

For RRR analyses, sub-sets of polyphenol metabolites were pre-selected using variable selection methods: RRR-VIP and LASSO. Previous studies have already observed that most of the selected polyphenols by the two methods here are associated with polyphenol-rich foods or food groups. Hesperetin and naringenin are known abundant polyphenols in citrus fruits [46], and 3,4-dihydroxyphenlylacetic acid (3,4-DHPAA) and 3-hydroxyphenlylacetic acid (3-HPAA) are two metabolites formed from hesperetin and naringenin by the colonic microbiota [47]. Hydroxytyrosol, thyrosol and 3,4-DHPAA are predominant polyphenols in olives [48]. Caffeic acid and chlorogenic acid are two major polyphenols from coffee [49], which are metabolized into 3,4-DHPAA, protocatechuic acid, m-coumaric acid, and vanillic acid by the gut microbiota [50]. Gallic acid ethyl ester and resveratrol are the main polyphenols in red wine [48]. These selected polyphenols included all polyphenols with high coefficients in univariate comparisons. However, selected polyphenol metabolites differed by method, even though the first one or two metabolites were common in both methods. The LASSO method selected more polyphenol metabolites that were negatively associated with polyphenol-rich food intake. It seems that the variable selection using LASSO may be more

affected by other factors, such as dietary patterns, while the RRR-VIP method may focus on explaining polyphenol-rich food intake itself. Despite this difference in selected polyphenol metabolites, both methods performed equally well in explaining polyphenol-rich food intake, and both methods were overall equally efficient. Future studies may again compare the performance of both methods in different study settings.

The patterns of urinary polyphenol metabolites in this study better explained acute intake assessed by 24-HDR than habitual intake assessed by DQ, and performed equally bad as any single polyphenol metabolite (Table 4). A review paper [32] suggested that urinary polyphenol metabolites were useful as biomarkers for recent intake (12–72 h) based on results of some clinical trials and on knowledge on their pharmacokinetic properties (median half-life of 2.8 h) [51]. However, relatively high stability over time has been observed for a number of polyphenol metabolites, and this is most likely explained by the frequent consumption of their main food sources [33]. This is what is observed here for coffee and wine, and this also explains the correlations observed with DQ data. For other polyphenol metabolites less frequently consumed, reproducibility in urine may be lower, and this should largely explain the lower correlation of metabolite patterns with DQ data when compared to 24-HDR data. Therefore, polyphenol metabolite patterns could be used as biomarkers for acute dietary intake of polyphenol-containing foods, or for the regular intake of more frequently consumed foods, such as coffee or wine.

The strength of this study includes, first, its statistical design/strategy, which can be easily applied to other "-omics" datasets to identify potential biomarker patterns that can serve as dietary exposure markers. Second, the availability of 24-h urine samples offered additional advantages for the accurate assessment of polyphenol metabolites over spot urine or plasma samples, which are mainly available in most other cohort studies. However, our study had also some limitations. The study design or urinary polyphenol metabolites used in this study did not allow the identification of biomarkers for habitual dietary intake, except for frequently consumed foods (coffee or wine), hence, further research is needed to identify longer-term biomarkers. In addition, this study was carried out in European populations, so the statistical algorithm for identifying polyphenol metabolite patterns should be adapted to other populations as well.

5. Conclusions

Urinary polyphenol metabolite patterns performed better or equally well as compared to any single best polyphenol metabolite biomarker for intakes of specific polyphenol-rich foods, especially for acute dietary intake or regular intake of frequently consumed foods. The algorithm developed using dimension reduction and variable selection could be easily extended to other metabolites, foods, and food constituents.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/8/796/s1, Table S1: Correlation coefficients between urinary polyphenols and intakes of whole main food groups from 24-HDR among total subjects (n = 475), Table S2: Correlation coefficients between urinary polyphenols and intakes of vegetable groups from 24-HDR among total subjects (n = 475). Table S3: Correlation coefficients between urinary polyphenols and intakes of fruit groups from 24-HDR among total subjects (n = 475). Table S4: Correlation coefficients between urinary polyphenols and intakes of non-alcoholic beverage groups from 24-HDR among total subjects (n = 475). Table S5: Correlation coefficients between urinary polyphenols and intakes of alcoholic beverage groups from 24-HDR among total subjects (n = 475). Table S6: Correlations coefficients and ROC AUCs of RRR scores of selected polyphenol (PP) metabolites with polyphenol-rich foods from 24-HDR and DQ in total subjects (n = 475).

Acknowledgments: We thank Bertrand Hémon for valuable help with the EPIC database. This work was supported by the European Union (NutriTech FP7-KBBE-2011-5 grant 289511) and the Wereld Kanker Onderzoek Fonds (WCRF NL 2012/604). The coordination of EPIC is financially supported by the European Commission (DG-SANCO) and the International Agency for Research on Cancer. The national cohorts are supported by Ligue Contre le Cancer, Institut Gustave Roussy, Mutuelle Générale de l'Education Nationale, Institut National de la Santé et de la Recherche Médicale (INSERM) (France); German Cancer Aid, German Cancer Research Center (DKFZ), Federal Ministry of Education and Research (BMBF), Deutsche Krebshilfe, Deutsches Krebsforschungszentrum and Federal Ministry of Education and Research (Germany); the Hellenic Health Foundation (Greece); Associazione Italiana per la Ricerca sul Cancro-AIRC-Italy and National Research Council (Italy).

Author Contributions: A.S. and P.F. designed the study (project conception and development of overall research plan); R.Z.-R. and D.A.: generated polyphenol metabolite data (incl. experiments); N.S.: took responsibility for dietary intake data and their interpretation; H.N. and N.A.: performed the statistical analyses; H.F. and P.F.: supervised the statistical analyses; H.N., H.F., N.A. and P.F.: interpreted the results, H.N.: drafted the manuscript, and P.F.: had primary responsibility for final content. A.A., F.M., M.-C.B.-R., A.F., H.B., T.K., R.S., A.T., A.N., M.K., D.P., V.P., R.T., F.R., M.S.d.M. and A.C. (EPIC investigators): conducted the research and took responsibility for the study design and data collection. All authors critically revised the manuscript for intellectual content and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Freedman, L.S.; Potischman, N.; Kipnis, V.; Midthune, D.; Schatzkin, A.; Thompson, F.E.; Troiano, R.P.; Prentice, R.; Patterson, R.; Carroll, R.; et al. A comparison of two dietary instruments for evaluating the fat-breast cancer relationship. *Int. J. Epidemiol.* **2006**, *35*, 1011–1021. [CrossRef] [PubMed]
2. Kipnis, V.; Midthune, D.; Freedman, L.; Bingham, S.; Day, N.E.; Riboli, E.; Ferrari, P.; Carroll, R.J. Bias in dietary-report instruments and its implications for nutritional epidemiology. *Public Health Nutr.* **2002**, *5*, 915–923. [CrossRef] [PubMed]
3. Ferrari, P.; Roddam, A.; Fahey, M.T.; Jenab, M.; Bamia, C.; Ocké, M.; Amiano, P.; Hjartåker, A.; Biessy, C.; Rinaldi, S.; et al. A bivariate measurement error model for nitrogen and potassium intakes to evaluate the performance of regression calibration in the European Prospective Investigation into Cancer and Nutrition study. *Eur. J. Clin. Nutr.* **2009**, *63*, S179–S187. [CrossRef] [PubMed]
4. Jenab, M.; Slimani, N.; Bictash, M.; Ferrari, P.; Bingham, S.A. Biomarkers in nutritional epidemiology: Applications, needs and new horizons. *Hum. Genet.* **2009**, *125*, 507–525. [CrossRef] [PubMed]
5. Kuhnle, G.G.C. Nutritional biomarkers for objective dietary assessment. *J. Sci. Food Agric.* **2012**, *92*, 1145–1149. [CrossRef] [PubMed]
6. Potischman, N.; Freudenheim, J.L. Biomarkers of nutritional exposure and nutritional status: An overview. *J. Nutr.* **2003**, *133*, 873S–874S. [PubMed]
7. O'Gorman, A.; Gibbons, H.; Brennan, L. Metabolomics in the identification of biomarkers of dietary intake. *Comput. Struct. Biotechnol. J.* **2013**, *4*, e201301004. [CrossRef] [PubMed]
8. Scalbert, A.; Brennan, L.; Manach, C.; Andres-Lacueva, C.; Dragsted, L.O.; Draper, J.; Rappaport, S.M.; van der Hooft, J.J.J.; Wishart, D.S. The food metabolome: A window over dietary exposure. *Am. J. Clin. Nutr.* **2014**, *99*, 1286–1308. [CrossRef] [PubMed]
9. Altorf-van der Kuil, W.; Brink, E.J.; Boetje, M.; Siebelink, E.; Bijlsma, S.; Engberink, M.F.; van't Veer, P.; Tomé, D.; Bakker, S.J.L.; van Baak, M.A. Identification of biomarkers for intake of protein from meat, dairy products and grains: A controlled dietary intervention study. *Br. J. Nutr.* **2013**, *110*, 810–822. [CrossRef] [PubMed]
10. Jin, Y.; Gordon, M.H.; Alimbetov, D.; Chong, M.F.-F.; George, T.W.; Spencer, J.P.E.; Kennedy, O.B.; Tuohy, K.; Minihane, A.-M.; Lovegrove, J.A. A novel combined biomarker including plasma carotenoids, vitamin C, and ferric reducing antioxidant power is more strongly associated with fruit and vegetable intake than the individual components. *J. Nutr.* **2014**, *144*, 1866–1872. [CrossRef] [PubMed]
11. Pérez-Jiménez, J.; Neveu, V.; Vos, F.; Scalbert, A. Systematic analysis of the content of 502 polyphenols in 452 foods and beverages: An application of the phenol-explorer database. *J. Agric. Food Chem.* **2010**, *58*, 4959–4969. [CrossRef] [PubMed]
12. Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287–306. [CrossRef] [PubMed]
13. Van Dam, R.M.; Naidoo, N.; Landberg, R. Dietary flavonoids and the development of type 2 diabetes and cardiovascular diseases: Review of recent findings. *Curr. Opin. Lipidol.* **2013**, *24*, 25–33. [CrossRef] [PubMed]
14. Grosso, G.; Micek, A.; Godos, J.; Pajak, A.; Sciacca, S.; Galvano, F.; Giovannucci, E.L. Dietary Flavonoid and Lignan Intake and Mortality in Prospective Cohort Studies: Systematic Review and Dose-Response Meta-Analysis. *Am. J. Epidemiol.* **2017**, *185*, 1304–1316. [CrossRef] [PubMed]
15. Grosso, G.; Godos, J.; Lamuela-Raventos, R.; Ray, S.; Micek, A.; Pajak, A.; Sciacca, S.; D'Orazio, N.; Del Rio, D.; Galvano, F. A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. *Mol. Nutr. Food Res.* **2017**, *61*. [CrossRef] [PubMed]

16. Pérez-Jiménez, J.; Hubert, J.; Hooper, L.; Cassidy, A.; Manach, C.; Williamson, G.; Scalbert, A. Urinary metabolites as biomarkers of polyphenol intake in humans: A systematic review. *Am. J. Clin. Nutr.* **2010**, *92*, 801–809. [CrossRef] [PubMed]
17. Ito, H.; Gonthier, M.-P.; Manach, C.; Morand, C.; Mennen, L.; Rémésy, C.; Scalbert, A. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. *Br. J. Nutr.* **2005**, *94*, 500–509. [CrossRef] [PubMed]
18. Mennen, L.I.; Sapinho, D.; Ito, H.; Bertrais, S.; Galan, P.; Hercberg, S.; Scalbert, A. Urinary flavonoids and phenolic acids as biomarkers of intake for polyphenol-rich foods. *Br. J. Nutr.* **2006**, *96*, 191–198. [CrossRef] [PubMed]
19. Edmands, W.M.; Ferrari, P.; Rothwell, J.A.; Rinaldi, S.; Slimani, N.; Barupal, D.K.; Biessy, C.; Jenab, M.; Clavel-Chapelon, F.; Fagherazzi, G.; et al. Polyphenol metabolome in human urine and its association with intake of polyphenol-rich foods across European countries. *Am. J. Clin. Nutr.* **2015**, *102*, 905–913. [CrossRef] [PubMed]
20. Zamora-Ros, R.; Achaintre, D.; Rothwell, J.A.; Rinaldi, S.; Assi, N.; Ferrari, P.; Leitzmann, M.; Boutron-Ruault, M.-C.; Fagherazzi, G.; Auffret, A.; et al. Urinary excretions of 34 dietary polyphenols and their associations with lifestyle factors in the EPIC cohort study. *Sci. Rep.* **2016**, *6*, 26905. [CrossRef] [PubMed]
21. Riboli, E. The European Prospective Investigation into Cancer and Nutrition (EPIC): Plans and progress. *J. Nutr.* **2001**, *131*, S170–S175.
22. Slimani, N.; Kaaks, R.; Ferrari, P.; Casagrande, C.; Clavel-Chapelon, F.; Lotze, G.; Kroke, A.; Trichopoulos, D.; Trichopoulou, A.; Lauria, C.; et al. European Prospective Investigation into Cancer and Nutrition (EPIC) calibration study: Rationale, design and population characteristics. *Public Health Nutr.* **2002**, *5*, 1125. [CrossRef] [PubMed]
23. Slimani, N.; Bingham, S.; Runswick, S.; Ferrari, P.; Day, N.E.; Welch, A.A.; Key, T.J.; Miller, A.B.; Boeing, H.; Sieri, S.; et al. Group level validation of protein intakes estimated by 24-h diet recall and dietary questionnaires against 24-h urinary nitrogen in the European Prospective Investigation into Cancer and Nutrition (EPIC) calibration study. *Cancer Epidemiol. Biomark. Prev.* **2003**, *12*, 784–795.
24. Slimani, N.; Fahey, M.; Welch, A.A.; Wirfält, E.; Stripp, C.; Bergström, E.; Linseisen, J.; Schulze, M.B.; Bamia, C.; Chloptsios, Y.; et al. Diversity of dietary patterns observed in the European Prospective Investigation into Cancer and Nutrition (EPIC) project. *Public Health Nutr.* **2002**, *5*, 1311–1328. [CrossRef] [PubMed]
25. Slimani, N.; Casagrande, C.; Nicolas, G.; Freisling, H.; Huybrechts, I.; Ocké, M.C.; Niekerk, E.M.; van Rossum, C.; Bellemans, M.; De Maeyer, M.; et al. The standardized computerized 24-h dietary recall method EPIC-Soft adapted for pan-European dietary monitoring. *Eur. J. Clin. Nutr.* **2011**, *65*, S5–S15. [CrossRef] [PubMed]
26. Achaintre, D.; Buleté, A.; Cren-Olivé, C.; Li, L.; Rinaldi, S.; Scalbert, A. Differential Isotope Labeling of 38 Dietary Polyphenols and Their Quantification in Urine by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry. *Anal. Chem.* **2016**, *88*, 2637–2644. [CrossRef] [PubMed]
27. Dempster, A.P. Maximum Likelihood from Incomplete Data via the EM Algorithm on JSTOR. Available online: http://www.jstor.org/stable/2984875?seq=1#page_scan_tab_contents (accessed on 11 December 2015).
28. Mehmood, T.; Martens, H.; Sæbø, S.; Warringer, J.; Snipen, L. A Partial Least Squares based algorithm for parsimonious variable selection. *Algorithms Mol. Biol.* **2011**, *6*, 27. [CrossRef] [PubMed]
29. Tibshirani, R. Regression shrinkage and selection via the lasso: A retrospective. *J. R. Stat. Soc. Ser. B Stat. Methodol.* **2011**, *73*, 273–282. [CrossRef]
30. Chong, I.-G.; Jun, C.-H. Performance of some variable selection methods when multicollinearity is present. *Chemom. Intell. Lab. Syst.* **2005**, *78*, 103–112. [CrossRef]
31. Zamora-Ros, R.; Knaze, V.; Rothwell, J.A.; Hémon, B.; Moskal, A.; Overvad, K.; Tjønneland, A.; Kyrø, C.; Fagherazzi, G.; Boutron-Ruault, M.C.; et al. Dietary polyphenol intake in europe: The european prospective investigation into cancer and nutrition (EPIC) study. *Eur. J. Nutr.* **2016**, *55*, 1359–1375. [CrossRef] [PubMed]
32. Spencer, J.P.E.; Abd El Mohsen, M.M.; Minihane, A.-M.; Mathers, J.C. Biomarkers of the intake of dietary polyphenols: Strengths, limitations and application in nutrition research. *Br. J. Nutr.* **2008**, *99*, 12–22. [CrossRef] [PubMed]
33. Zamora-Ros, R.; Touillaud, M.; Rothwell, J.A.; Romieu, I.; Scalbert, A. Measuring exposure to the polyphenol metabolome in observational epidemiologic studies: Current tools and applications and their limits. *Am. J. Clin. Nutr.* **2014**, *100*, 11–26. [CrossRef] [PubMed]

34. Noroozi, M.; Burns, J.; Crozier, A.; Kelly, I.E.; Lean, M.E. Prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion. *Eur. J. Clin. Nutr.* **2000**, *54*, 143–149. [CrossRef] [PubMed]
35. Erlund, I.; Silaste, M.L.; Alfthan, G.; Rantala, M.; Kesäniemi, Y.A.; Aro, A. Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high or low in fruit and vegetables. *Eur. J. Clin. Nutr.* **2002**, *56*, 891–898. [CrossRef] [PubMed]
36. Nielsen, S.E.; Freese, R.; Kleemola, P.; Mutanen, M. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. *Cancer Epidemiol. Biomark. Prev.* **2002**, *11*, 459–466.
37. Krogholm, K.S.; Haraldsdóttir, J.; Knuthsen, P.; Rasmussen, S.E. Urinary total flavonoid excretion but not 4-pyridoxic acid or potassium can be used as a biomarker for the intake of fruits and vegetables. *J. Nutr.* **2004**, *134*, 445–451. [PubMed]
38. Hodgson, J.M.; Chan, S.Y.; Puddey, I.B.; Devine, A.; Wattanapenpaiboon, N.; Wahlqvist, M.L.; Lukito, W.; Burke, V.; Ward, N.C.; Prince, R.L.; et al. Phenolic acid metabolites as biomarkers for tea- and coffee-derived polyphenol exposure in human subjects. *Br. J. Nutr.* **2004**, *91*, 301–306. [CrossRef] [PubMed]
39. Zamora-Ros, R.; Urpí-Sardà, M.; Lamuela-Raventós, R.M.; Estruch, R.; Martínez-González, M.A.; Bulló, M.; Arós, F.; Cherubini, A.; Andres-Lacueva, C. Resveratrol metabolites in urine as a biomarker of wine intake in free-living subjects: The PREDIMED Study. *Free Radic. Biol. Med.* **2009**, *46*, 1562–1566. [CrossRef] [PubMed]
40. Mennen, L.I.; Sapinho, D.; Ito, H.; Galan, P.; Hercberg, S.; Scalbert, A. Urinary excretion of 13 dietary flavonoids and phenolic acids in free-living healthy subjects—Variability and possible use as biomarkers of polyphenol intake. *Eur. J. Clin. Nutr.* **2008**, *62*, 519–525. [CrossRef] [PubMed]
41. Hu, F.B. Dietary pattern analysis: A new direction in nutritional epidemiology. *Curr. Opin. Lipidol.* **2002**, *13*, 3–9. [CrossRef] [PubMed]
42. Izenman, A.J. Reduced-rank regression for the multivariate linear model. *J. Multivar. Anal.* **1975**, *5*, 248–264. [CrossRef]
43. Hoffmann, K. Application of a New Statistical Method to Derive Dietary Patterns in Nutritional Epidemiology. *Am. J. Epidemiol.* **2004**, *159*, 935–944. [CrossRef] [PubMed]
44. Sugimoto, M.; Kawakami, M.; Robert, M.; Soga, T.; Tomita, M. Bioinformatics Tools for Mass Spectroscopy-Based Metabolomic Data Processing and Analysis. *Curr. Bioinform.* **2012**, *7*, 96–108. [CrossRef] [PubMed]
45. Boccard, J.; Veuthey, J.-L.; Rudaz, S. Knowledge discovery in metabolomics: An overview of MS data handling. *J. Sep. Sci.* **2010**, *33*, 290–304. [CrossRef] [PubMed]
46. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P. E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892. [CrossRef] [PubMed]
47. Gao, K.; Xu, A.; Krul, C.; Venema, K.; Liu, Y.; Niu, Y.; Lu, J.; Bensoussan, L.; Seeram, N.P.; Heber, D.; et al. Of the Major Phenolic Acids Formed during Human Microbial Fermentation of Tea, Citrus, and Soy Flavonoid Supplements, Only 3,4-Dihydroxyphenylacetic Acid Has Antiproliferative Activity. *J. Nutr.* **2006**, *136*, 52–57. [PubMed]
48. Neveu, V.; Perez-Jiménez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D.; et al. Phenol-Explorer: An online comprehensive database on polyphenol contents in foods. *Database Oxf.* **2010**, *2010*, bap024. [CrossRef] [PubMed]
49. Del Rio, D.; Stalmach, A.; Calani, L.; Crozier, A. Bioavailability of coffee chlorogenic acids and green tea flavan-3-ols. *Nutrients* **2010**, *2*, 820–833. [CrossRef] [PubMed]
50. Gonthier, M.-P.; Remesy, C.; Scalbert, A.; Cheynier, V.; Souquet, J.-M.; Poutanen, K.; Aura, A.-M. Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomed. Pharmacother.* **2006**, *60*, 536–540. [CrossRef] [PubMed]
51. Rothwell, J.A.; Urpi-Sarda, M.; Boto-Ordoñez, M.; Llorach, R.; Farran-Codina, A.; Barupal, D.K.; Neveu, V.; Manach, C.; Andres-Lacueva, C.; Scalbert, A. Systematic analysis of the polyphenol metabolome using the Phenol-Explorer database. *Mol. Nutr. Food Res.* **2016**, *60*, 203–211. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Anti-Diabetic Effects of Phenolic Extract from Rambutan Peels (*Nephelium lappaceum*) in High-Fat Diet and Streptozotocin-Induced Diabetic Mice

Qingyu Ma, Yan Guo, Liping Sun and Yongliang Zhuang *

Yunnan Institute of Food Safety, Kunming University of Science and Technology, No. 727 South Jingming Road, Kunming 650500, China; mqy0323@hotmail.com (Q.M.); guoyan8090@hotmail.com (Y.G.); lpsun@kmust.edu.cn (L.S.)

* Correspondence: ylzhuang@kmust.edu.cn; Tel./Fax: +86-871-6592-0216

Received: 6 June 2017; Accepted: 20 July 2017; Published: 26 July 2017

Abstract: Recent studies have shown that rambutan peel phenolic (RPP) extract demonstrate high antioxidant and antiglycation activities in vitro and in vivo. This study further evaluated the anti-diabetic activity of RPP in a mouse model of Type II diabetes induced by streptozotocin combined with high-fat diet. Results showed that RPP increased the body weight and reduced the fasting blood glucose level of the diabetic mice. RPP significantly reduced the serum levels of total cholesterol, triglyceride, creatinine, and glycated serum protein in diabetic mice in a dose-dependent manner. Glycogen content in mice liver was recovered by RPP, which further increased the activity of superoxide dismutase and glutathione peroxidase and reduced lipid peroxidation in diabetic mice. Histological analysis showed that RPP effectively protected the tissue structure of the liver, kidney, and pancreas. In addition, RPP decreased the mesangial index and inhibited the expression of TGF-β in the kidney of diabetic mice.

Keywords: rambutan peels; phenolics; anti-diabetic; hepatic glycogen; histological analysis; immunohistochemical

---

1. Introduction

Diabetes mellitus (DM) is currently a major health problem worldwide. DM is the most common chronic disease characterized by elevated blood glucose levels [1]. Long-term exposure to high blood glucose levels results in production of reactive oxygen species. Oxidative stress is one of the main mechanisms of progression of diabetes and actively leads to cellular injury that precedes the onset of many diabetic complications [2]. Moreover, hyperglycemia is involved in the development of microvascular and macrovascular complications, which are the major causes of diabetes-related morbidity and mortality [3].

Researchers are developing a number of oral medicines to treat diabetes. However, these drugs demonstrate significant side effects, including weight gain and gastrointestinal distress. Therefore, finding new potential natural products that prevent DM is necessary [4]. Phenolic compounds are important secondary plant metabolites that determine the sensory and nutritional qualities of fruits, vegetables, and other plant products. Phenolics have received attention in recent years because of their antioxidant [5], antimicrobial [6], antimelanogenesis [7], hepatoprotective [8] and anti-inflammatory [9] effects. Studies suggest that a large number of phenolic extracts from plants, including mulberry leaf [10], *Pistachia lentiscus* L. leaves from Algeria [11], *Astilboides tabularis* [12], mate tea [13], Korean sorghum [14], *Pongamia pinnata* Pierreseeds [15], and *Pseuduvaria monticola* bark [16], demonstrate anti-diabetic properties.

We previously extracted crude rambutan phenolic peel (RPP) extract through microwave-assisted extraction, wherein the operating parameters were optimized. Our study showed that RPP possesses

a potent free radical scavenging activity due to its phenolic contents [17]. Crude RPP extracts were purified using NKA-9 resin adsorption technology. After purification, phenolic content, along with some important phenolic compounds, such as geraniin (122.18 mg/g dry weight (dw)), procyanidin trimers (4.06 mg/g dw), procyanidin dimmers (11.60 mg/g dw), corilagin (7.56 mg/g dw), catechin (9.80 mg/g dw), and ellagic acid (9.31 mg/g dw), were enriched; additionally, the antioxidant and antiglycation activity in vitro were increased [18]. Purified RPP exerts high inhibitory oxidative stress on H_2O_2-induced HepG2 cells and anti-aging activity induced by D-galactose in vivo. Moreover, RPP effectively provides protection against D-gal-induced liver and kidney tissue damage in mice [19].

On the basis of the above results, we speculated that purified RPP can reduce blood glucose levels. However, no study has been conducted to scientifically prove the anti-diabetic activity of RPP. This study thus aims to demonstrate the anti-diabetic activity of RPP in animal models of diabetes induced by streptozotocin (STZ) combined with high-fat diet.

2. Materials and Methods

2.1. Materials and Reagents

Purified RPP sample was prepared as previously described [18]. High-fat diets (45% fat) were purchased from Research Diets Inc. (New Brunswich, NJ, USA). Dimethylbiguanide hydrochloride (DMBG) was purchased from Sino-American Shanghai Squibb Pharmaceuticals Ltd. (Shanghai, China). STZ was purchased from Sigma-Aldrich (St. Louis, MO, USA). Commercial assay kits for creatinine (CRE), glycated serum protein (GSP), total cholesterol (TC), total triglycerides (TG), total protein (TP), hepatic glycogen (GC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malonaldehyde (MDA) were obtained from Nanjing Jiancheng Biological Engineering Institute Co., Ltd. (Nanjing, China).

2.2. Experimental Animals

Male Institute of Cancer Research (ICR) mice (weighing 18–22 g) were provided by the Kunming Medical University Animal Research Center (Kunming, China). All mice were acclimatized for 7 days to the conditions of the animal room (ambient temperature of 20–25 °C and a 12 h-dark/12 h-light cycle) and provided with free access to standard pellet diets and water. All animal experiments were performed in strict accordance with the animal experimentation guidelines approved by the Animal Care and Use Committee of our Institute.

2.3. Experimental Design

2.3.1. Modeling Method

After a week-long adaptive fitness program, all mice were fasted for 6 h, and eight mice were randomly selected as members of the normal group (NG). The NG mice were injected with 0.06 mL of saline, whereas the other mice were intraperitoneally injected with 0.06 mL of STZ at a dose of 50 mg/kg body weight (bw). This treatment was repeated at same time for 3 days. Afterward, the ordinary feed was changed into high-fat diet. The mice fed with high-fat diet were followed up for 14 days. The fasting blood glucose level of these mice was measured, and those showing a fasting blood glucose level higher than 11.1 mmol/L were considered hyperglycemic.

The hyperglycemic mice were randomly divided into five groups, each group consisting of eight animals; these groups were the positive control group (PG, 10 mg/kg bw DMBG taken orally daily), Model group (MG, saline taken orally daily), low-dose group (RPP-L, 50 mg/kg bw RPP taken orally daily), middle-dose group (RPP-M, 100 mg/kg bw RPP taken orally daily), and high-dose group (RPP-H, 200 mg/kg bw RPP taken orally daily). The mice were weighed to adjust the oral doses according to changes in their weight. The fasting blood glucose level of the mice was measured from

the tail veins by using a glucometer (Bayer HealthCare LLC, Bayer, IN, USA), and the readings were recorded weekly for 5 weeks.

2.3.2. Biochemical Assays

Serum was collected from blood via centrifugation at 2000 r/min for 15 min at a temperature below 5 °C. Livers, kidneys, and pancreas were collected, and floating blood was washed out with ice-cold physiological saline.

Serum concentrations of CRE, GSP, TC, and TG were measured on an automatic biochemical analyzer (RaytoChemray 240, Shenzhen Rayto Co., Ltd., Shenzhen, China) by using commercial kits.

HG concentration was measured using a colorimetric method, whereas TP of liver was measured using the Bradford method. The levels of hepatic SOD, GSH-Px, and MDA were determined using commercial assay kits. All of the experimental procedures were performed strictly in accordance with the kit instructions.

2.3.3. Histopathological and Immunohistochemical Analyses of Tissues

Histopathological Analysis of Tissues

Tissues samples were obtained from liver, kidney, and pancreas, fixed in 10% buffered formalin, dehydrated in alcohol, and embedded in paraffin. Paraffin sections (2–3 μm thick) were cut and stained with hematoxylin-eosin (HE). Histopathological changes in the liver, kidney, and pancreas were visualized using an Olympus DP70 Digital Camera System at 200× magnification.

The 2–3 μm-thick paraffin sections of the kidney were cut and stained with Periodic Acid-Schiff (PAS). The condition of the lesion in glomeruli and interstitial tubules was observed under an Olympus DP70 Digital Camera System at 200× magnification.

Immunohistochemical Analysis of Tissues

Paraffin sections (2–3 μm thick) of kidney were blocked with 5% BSA solution after the process of microwave repair antigen. Subsequently, 100 μL of transforming growth factor-β1 (TGF-β1, 1:100) antibody was added into each slice at 4 °C overnight. After washing with tap water several times, 100 μL of sheep anti-mouse/rabbit IgG polymer was dropped on each slice, which was placed at room temperature for 15 min and then washed with phosphate buffer saline three times for 3 min each round. After being stained with diaminobenzidine, the slices were encapsulated in neutral resin film following conventional dewatering. The integral optical density of TGF-β1expression region was visualized with an Olympus DP70 Digital Camera System at 200× magnification and then measured using an image-pro Insight analysis software (Media cybernetics Inc., Rockville, MD, USA).

2.4. Statistical Analysis

Experimental data were presented as means ± SD (n = 8). Statistical significance of the difference between groups was detected using SPSS19.0 software (SPSS Inc., Chicago, IL, USA). p Values of <0.05 indicated statistical significance.

3. Results and Discussion

STZ produces oxygen free radicals in the body, resulting in selective pancreatic islet β-cell cytotoxicity and increased blood glucose level. Low-dose STZ induced metabolic characteristics of the human Type II diabetic mellitus (T2DM) [10]. Researchers have developed an animal model fed with low-dose STZ combined with high-fat diet to evaluate the effects of natural products on T2DM [20]. This model is potentially useful in studying the anti-diabetic properties of natural compounds of plant origin. In this study, mice with T2DM induced by high-fat diet combined with low-dose STZ injection were used to evaluate the anti-diabetic activity of RPP.

3.1. Body Weight

STZ-induced diabetes is characterized by a severe loss in body weight resulting from increased muscle destruction or degradation of structural proteins [21]. Compared with the body weight of NG mice, that of MG mice significantly decreased by 19.17% (Figure 1). When the diabetic mice were treated with RPP, their body weights improved. The body weight of RPP-M mice did not differ from that of PG mice ($p > 0.05$), the body weight of which was significantly higher than that of MG mice ($p < 0.05$). The body weight of RPP-H group did not differ with that of NG group ($p < 0.05$). These results indicated that RPP prevents body weight loss by controlling muscle wasting. Our results are consistent with those of a previous study [21].

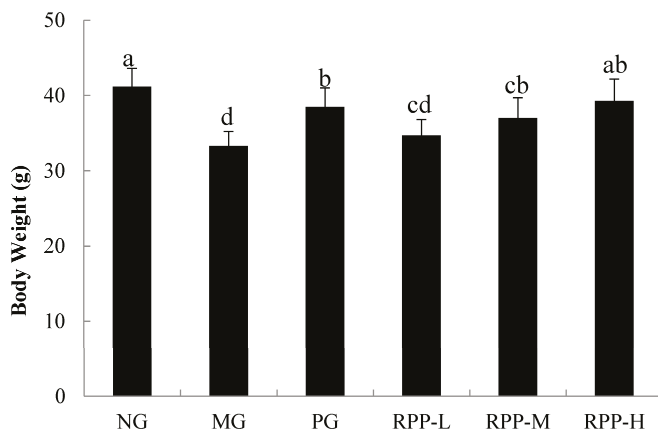


Figure 1. Effect of rambutan peel phenolic (RPP) on body weight of mice after 5 weeks. NG: Normal group; MG: streptozotocin (STZ), Model group; PG: STZ, DMBG 10 mg/kg; RPP-L: STZ, 50 mg/kg RPP; RPP-M: STZ, 100 mg/kg RPP; RPP-H: STZ, 200 mg/kg RPP. Different lower-case letters indicate significant differences ($p < 0.05$).

3.2. Fasting Blood Glucose (FBG)

High FBG is a key indicator in diabetic mice. After STZ injection, FBG levels in MG mice were significantly higher than those in NG mice ($p < 0.05$) (Figure 2), indicating that a T2DM model was successfully built through STZ injection. These results suggest that RPP effectively inhibited the increase in FBG in T2DM, and the inhibitory activity increased with increased RPP concentration. RPP-H had stronger inhibitory ability than PG, indicating that RPP noticeably inhibited FBG in diabetic mice. Studies have shown that the products demonstrating antioxidant and antiglycation activities effectively inhibit the increase in FBG. Mehenni et al. studied that gallic acid, catechin and ellagic acid in *Pistacia lentiscus* were key compositions to regulate glucose in diabetic rat [22]. Thus, as we previously reported [17–19], RPP were rich in catechin and ellagic acid, so the FBG inhibitory activity of RPP was due to the phenolic compounds with high antioxidant and antiglycation activities.

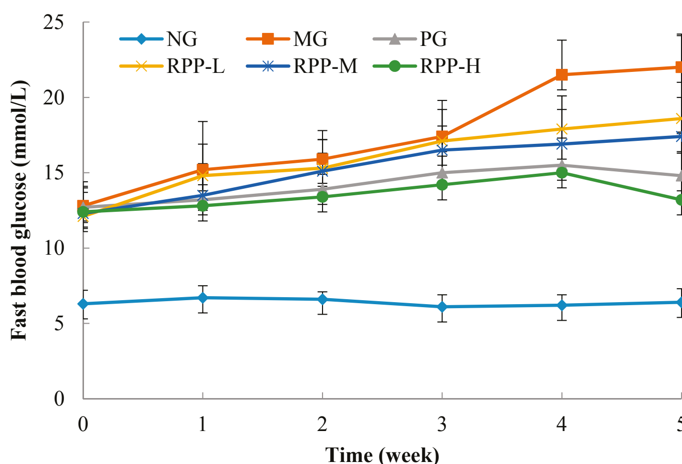


Figure 2. Effect of RPP on fast blood glucose level of mice. NG: Normal group; MG: STZ, Model group; PG: STZ, DMBG 10 mg/kg; RPP-L: STZ, 50 mg/kg RPP; RPP-M: STZ, 100 mg/kg RPP; RPP-H: STZ, 200 mg/kg RPP.

3.3. Serum Biochemical Indicators

TC refers to the sum of all lipoprotein cholesterols in the blood and is an important indicator in analysis of blood lipid in clinical practice. The high level of cholesterol can lead to atherosclerosis, diabetes, and symptoms of kidney disease. TG is a type of fatty acid molecule consisting of long-chain fatty acids and glycerol [23]. Part of the sugar can be converted into TG by the liver in vivo. Moreover, high levels of TG can lead to high blood pressure, pancreatitis, in aggravated hepatitis, or other injuries. As shown in Table 1, TC and TG levels in STZ-induced MG mice considerably increased compared with the NG level ($p < 0.05$). The serum TC and TG levels in RPP groups significantly decreased in a dose-dependent manner. The TC and TG levels in RPP-H mice did not significantly differ from those in NG mice ($p > 0.05$).

Table 1. Effect of RPP on biochemical indicators of mice serum.

Group	TC (mmol/L)	TG (mmol/L)	CRE (μmol/L)	GSP (mmol/L)
NG	5.90 ± 0.31d	1.43 ± 0.11c	10.62 ± 1.53d	7.14 ± 0.43d
MG	8.57 ± 0.43a	2.27 ± 0.13a	22.78 ± 1.96a	14.08 ± 1.12a
PG	6.28 ± 0.26c	1.53 ± 0.09c	13.93 ± 0.98c	7.93 ± 0.62d
RPP-L	7.12 ± 0.11bc	1.74 ± 0.11b	20.50 ± 2.04a	13.14 ± 0.86b
RPP-M	6.37 ± 0.35c	1.54 ± 0.10c	16.60 ± 1.14b	10.65 ± 0.91bc
RPP-H	6.01 ± 0.26cd	1.46 ± 0.12c	12.09 ± 0.85c	8.72 ± 0.78c

NG: Normal group; MG: STZ, Model group; PG: STZ, DMBG 10 mg/kg; RPP-L: STZ, 50 mg/kg RPP; RPP-M: STZ, 100 mg/kg RPP; RPP-H: STZ, 200 mg/kg RPP; TC: total cholesterol, TG: total triglycerides; CRE: creatinine; GSP: glycated serum protein. Different lower-case letters indicate significant differences ($p < 0.05$).

CRE is an indicator of the toxin content of the blood and an important indicator of diabetic nephropathy [24]. As shown in Table 1, CRE content significantly increased ($p < 0.05$) in MG group and approximately twice that in NG mice. RPP obviously dose-dependently inhibited the increase in CRE content in diabetic mice. Relative to the CRE level in MG mice, that in RPP-M and RPP-H mice decreased by 27.13% and 46.93%, respectively.

GSP is produced during plasma protein and glucose enzyme saccharification. High levels of blood glucose lead to the production of high GSP levels in a positive correlated manner. GSP levels

reflect the average blood glucose levels within 1–3 weeks before [25]. Table 1 shows that the GSP contents in MG mice were significantly higher than those in NG mice ($p < 0.05$), which proved high blood glucose increased the production of GSP in mice. RPP and DMBG significantly reduced the GSP level in STZ-induced diabetic mice ($p < 0.05$) in a dose-dependent manner. Compared with that in MG mice, the GSP levels in RPP-M, RPP-L, and RPP-H mice decreased by 6.68%, 24.36%, and 38.07%, respectively. The change of GSP content in the serum of diabetic mice after treatment with RPP was same to the change of FBG (Figure 2). The activity of RPP was due to the high antioxidant and antiglycation activities [17–19]. Our results were similar to the previous study [24].

3.4. Biochemical Indicators in the Liver

TP level decreased in the liver of diabetic mice (Table 2). Compared with that in NG mice, the protein level in MG mice decreased by 23.53% ($p < 0.05$). Protein level in the liver of diabetic mice improved after treatment with RPP. The protein levels in RPP-M, RPP-H, and PG mice did not significantly differ from that in NG mice ($p > 0.05$). This result was obtained due to the distinct metabolic alterations that led to a negative nitrogen balance, enhancing proteolysis and reducing protein synthesis [26].

Table 2. Effect of RPP on biochemical indicators in mice liver.

Group	TP (g/g Liver)	GC (mg/g Liver)	SOD (U/mg)	GSH-Px (U/mg)	MDA (nmol/mg)
NG	0.17 ± 0.01a	32.37 ± 6.15a	316.19 ± 24.06a	105.11 ± 9.02a	1.18 ± 0.18c
MG	0.13 ± 0.02b	16.58 ± 4.76c	214.26 ± 17.10d	67.13 ± 7.00b	1.74 ± 0.37a
PG	0.16 ± 0.01a	27.70 ± 3.92b	305.60 ± 31.74a	101.58 ± 12.10a	1.36 ± 0.07b
RPP-L	0.13 ± 0.01b	19.62 ± 4.11bc	256.82 ± 31.63c	85.18 ± 13.64ab	1.70 ± 0.16a
RPP-M	0.16 ± 0.01a	22.22 ± 5.26b	263.69 ± 25.11bc	97.76 ± 6.30a	1.45 ± 0.24b
RPP-H	0.16 ± 0.01a	25.05 ± 4.71b	297.65 ± 23.29b	101.83 ± 10.60a	1.17 ± 0.16c

NG: Normal group; MG: STZ, Model group; PG: STZ, DMBG 10 mg/kg; RPP-L: STZ, 50 mg/kg RPP; RPP-M: STZ, 100 mg/kg RPP; RPP-H: STZ, 200 mg/kg RPP; TP: total protein; GC: hepatic glycogen; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; MDA: malonaldehyde. Different lower-case letters indicate significant differences ($p < 0.05$).

GC is a type of macromolecular polysaccharide composed of glucose units and is mainly stored in the liver and muscles as standby energy. GC level in various tissues directly reflects insulin activity [27]. In this study, the GC content of the liver of the diabetic mice was markedly reduced. As shown in Table 2, the GC level in MG mice significantly decreased by 48.78% ($p < 0.05$) compared with that in NG mice. RPP dose-dependently increased the GC level in the liver of diabetic rats. The increase in GC levels under the three RPP doses did not significant differ ($p > 0.05$). RPP-M and RPP-H had no significant difference with PG mice ($p > 0.05$).

Oxidative stress is one of the main mechanisms of progression of diabetes and actively leads to cellular injury that can precede the onset of many diabetic complications [28]. Long-term exposure to high glucose levels results in increased production of reactive oxygen species. Oxidative stress is generally considered a causative factor in the development of insulin resistance and diabetic complications .This study further evaluated the protective effect of RPP on antioxidant enzyme and liver lipid peroxide in mice.

SOD and GSH-Px enzymes are important in enzymatic defense system in vivo. SOD converts superoxide radicals into hydrogen peroxide, whereas GSH-Px converts hydrogen peroxide into other compounds in the presence of glutathione [29]. Excessive production of reactive oxygen in the serum of diabetic animals is possibly due to the observed marked reduction in SOD and GSH-Px concentrations. As shown in Table 2, SOD activity in MG mice decreased by 32.24% compared with that in NG mice. This result suggests that SOD activity is disrupted in diabetic mice. The SOD activity in PG mice did not significantly differ with that in NG mice ($p > 0.05$). RPP increased the SOD activity, which was significantly higher in RPP-H mice than in MG mice ($p < 0.05$), reaching 94.14% that of NG. The GSH-Px

activity in MG mice decreased significantly compared with that in NG mice ($p < 0.05$). RPP recovered the GSH-Px activity. At the low dose of RPP, the GSH-Px activity in RPP-L did not significantly differ with that in NG ($p > 0.05$). Our previous studies showed RPP had high antioxidant activities and protected antioxidant enzymes of D-gal-induced aging mice in vivo [17–19]. Therefore, the high antioxidant activity of RPP was a mechanism to protect the antioxidant enzyme activity from diabetic animals.

STZ induces severe oxidative stress in diabetic animals and possibly induces the peroxidation of polyunsaturated fatty acids, leading to the formation of MDA, which is a by-product of lipid peroxidation [30]. In this study, MDA content of MG mice significantly increased by 47.46% compared with that in NG mice. RPP inhibited the increase in MDA content in a dose-dependent manner. The MDA levels in RPP-H did not significantly differ from that in NG ($p > 0.05$). The result was similar to previous study [22]. It indicated that the antioxidant activity of RPP inhibited lipid peroxide in the liver of diabetic animals.

Regarding the literature available, catechin [31], geraniin [31], procyanidin [32] and ellagic acid [33] have been related to anti-diabetic activity, both in vivo and in vitro, and with the involvement of different action mechanisms. Our previous studies showed geraniin, procyanidin, catechin, and ellagic acid had high concentrations in RPP [19]. Therefore, anti-diabetic activity of RPP was due to its phenolic compounds.

4. Histopathology

4.1. HE staining of Liver

Figure 3a shows the normal hepatic architecture of the mice. The hepatocytes showed distinct cell borders, and the central vein showed a round nucleus, which is surrounded by abundant cytoplasm. In MG mice (Figure 3b), the STZ-induced diabetic mice showed mussy hepatic cords. The hepatic nucleus presented serious pathological damage. The intercellular space increased, and deterioration in terms of size and shape were serious. Other damages, including focal necrosis, congestion in central vein, and infiltration of lymphocytes, were also observed. The PG group (Figure 3c) showed a normal hepatic architecture, and changes in size and shape of the hepatic cells were not evident. RPP treatment alleviated the pathological damage in the experimental groups compared with that in MG mice. In RPP groups (Figure 3d–f), RPP demonstrated a dose-dependent protective effect on the STZ-induced diabetic mice. RPP-H apparently effectively alleviated the symptoms of focal necrosis, congestion in central vein, and infiltration of lymphocytes.

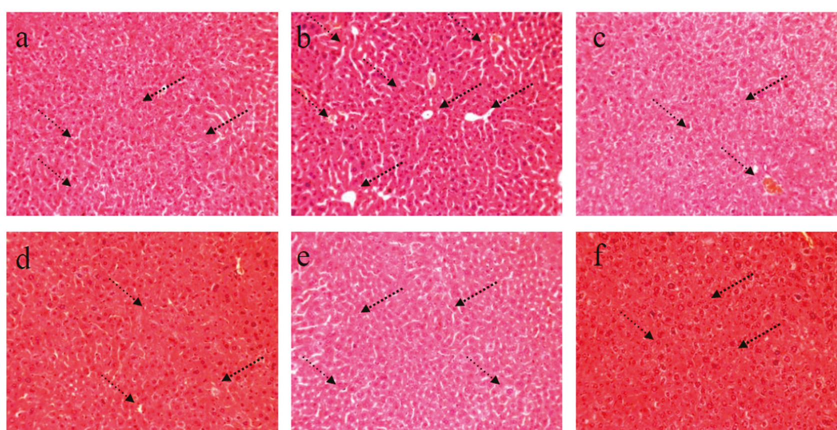


Figure 3. Effect of RPP on liver histology (H&E stain, 200×). (**a**) NG (Normal group); (**b**) MG (STZ, Model group); (**c**) PG (STZ, DMBG 10 mg/kg); (**d**) RPP-L(STZ, 50 mg/kg RPP); (**e**) RPP-M (STZ, 100 mg/kg RPP); (**f**) RPP-H (STZ, 200 mg/kg RPP).

4.2. HE Staining of Kidney

As shown in Figure 4a, the pathological tissue section of kidney revealed the distinct structure of both cortex and medulla in NG mice. The regular shape of glomeruli, renal tubule, and collecting duct was distinguished easily. The medulla displayed a distinctly ordered and packed arrangement. Moreover, symptoms of hemangiectasis, congestion, and inflammatory cell infiltration were not observed in the interstitial part. As presented in Figure 4b, the renal cortex and medulla of the STZ-induced diabetic MG mice showed varying degrees of atrophy. The cortex and medulla showed an architecture characterized by irregular distribution. The number of glomeruli declined obviously, and very serious glomerular sclerosis and expansion of kidney tubules were observed. The symptoms of inflammatory cell infiltration and congestion in central vein indicated a serious condition. Massive inflammatory cells infiltrated the glomeruli. The STZ-induced diabetic PG mice were treated with DMBG (Figure 4c). Glomerular degradation was alleviated to some degree. However, inflammatory cells still infiltrated the glomerular cells. Moreover, the medulla area is closely packed. In RPP experimental groups, diabetic nephropathy was relieved in different degrees after treatment with different RPP concentrations, especially the symptoms of congestion in central vein and inflammatory cell infiltration. This protective effect was dose dependent. RPP-H demonstrated the best effect on STZ-induced diabetic mice and restored the condition of the mice to nearly normal condition. This result indicated that RPP provided protection against STZ-induced kidney damage in diabetic mice.

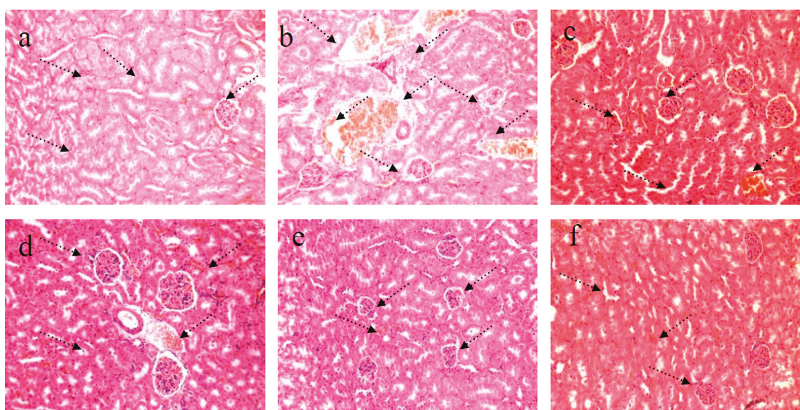


Figure 4. Effect of RPP on kidney histology (H&E stain, 200×). (**a**) NG (Normal group); (**b**) MG (STZ, Model group); (**c**) PG (STZ, DMBG 10 mg/kg); (**d**) RPP-L(STZ, 50 mg/kg RPP); (**e**) RPP-M (STZ, 100 mg/kg RPP); (**f**) RPP-H (STZ, 200 mg/kg RPP).

4.3. HE Staining of Pancreas

As shown in Figure 5a, the pancreatic cells in NG mice showed a compact and ordered arrangement, as well as displayed a regular shape. Moreover, intercellular spaces were distributed uniformly, and congestion in central vein was not obvious. In MG mice (Figure 4b), serious pathological damages, such as focal necrosis, congestion in central vein, and infiltration of lymphocytes, were observed. In addition, the pancreatic cells showed a seriously altered shape, and they showed very irregular distribution. The pathology of pancreatic tissue generally demonstrates the varying degrees of damages in STZ-induced diabetic mice. In PG mice, the shape and size of the pancreatic cells was relatively homogeneous and are orderly packed. Moreover, the conditions of focal necrosis and infiltration of lymphocytes improved obviously (Figure 5c). The alleviation of pancreatic pathological damages varied in a dose-dependent manner in RPP-treated groups. As shown in Figure 4d–f,

the protective effect of RPP-H was significantly better than that of RPP-L and RPP-M. In RPP-H mice, histological damage was notably mitigated and the conditions were restored to nearly the normal state.

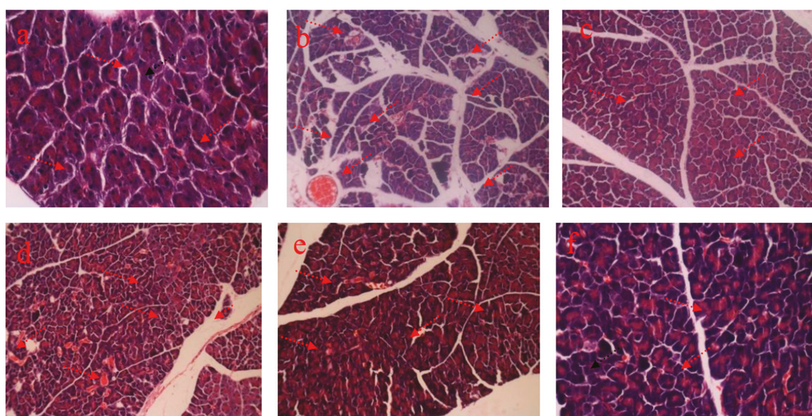


Figure 5. Effect of RPP on pancreas histology (H&E stain, 200×). (**a**) NG (Normal group); (**b**) MG (STZ, Model group); (**c**) PG (STZ, DMBG 10 mg/kg); (**d**) RPP-L(STZ, 50 mg/kg RPP); (**e**) RPP-M (STZ, 100 mg/kg RPP); (**f**) RPP-H (STZ, 200 mg/kg RPP).

4.4. PAS Staining of Kidney

After PAS staining, the cross-sectional area of glomeruli and glomeruli mesangial were analyzed by the Image-pro Insight analysis software (Media cybernetics Inc., Rockville, MD, USA). Mesangial matrix index, expressed as the ratio of the mesangial area to glomerular area, is an important indicator in evaluating kidney damage in diabetic mice. The kidney of STZ-induced diabetic mice showed glomerular enlargement and increased mesangial proliferation and mesangial index. As shown in Figure 6, the index in MG mice is obviously higher than that in NG mice. RPP treatment in each group mitigated histological damage and restored the mesangial index, thereby alleviating the symptoms of diabetes in mice. This protective effect was dose dependent. These results suggested that the effect of RPP on mesangial matrixis one of the key mechanisms in protecting the exterior of the kidney of STZ-induced diabetic mice.

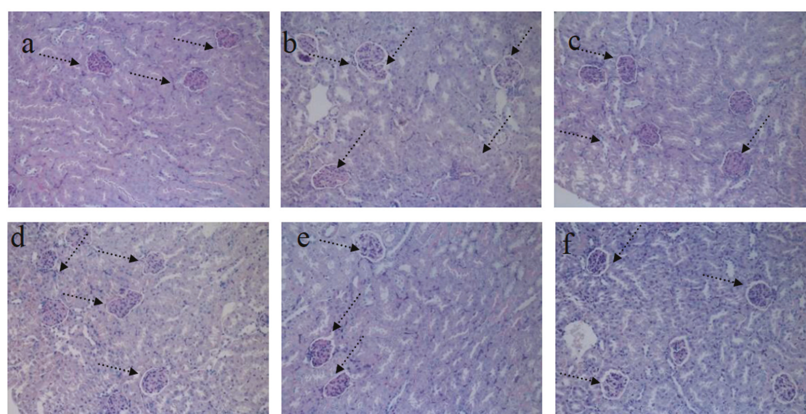


Figure 6. Effect of RPP on the mesangial index of kidney (PAS stain, 200×) (**a**) NG (Normal group); (**b**) MG (STZ, Model group); (**c**) PG (STZ, DMBG 10 mg/kg); (**d**) RPP-L(STZ, 50 mg/kg RPP); (**e**) RPP-M (STZ, 100 mg/kg RPP); (**f**) RPP-H (STZ, 200 mg/kg RPP).

4.5. TGF-β1 Staining of Kidney

TGF-β1 is an immunosuppressant that inhibits the growth of T cells and B cells.TGF-β1 plays an important role in regulating cell growth, differentiation, and immune function [34]. In this study, immunohistochemical staining of mice kidney with TGF-β1were evaluated by Image-pro insight analysis software (Media cybernetics Inc., Rockville, MD, USA). The results in Figure 7 show that TGF-β1 expression in MG mice increased markedly compared with that in NG mice. The RPP-treated groups showed reduced TGF-β1 expression. As RPP dose increases, the effect of RPP on TGF-β1 expression of mice kidney also increases. TGF-β1 expression in RPP-H did not significantly differ with that in NG. TGF-β1 expression is possibly another protective mechanism of RPP in STZ-induced diabetic mice.

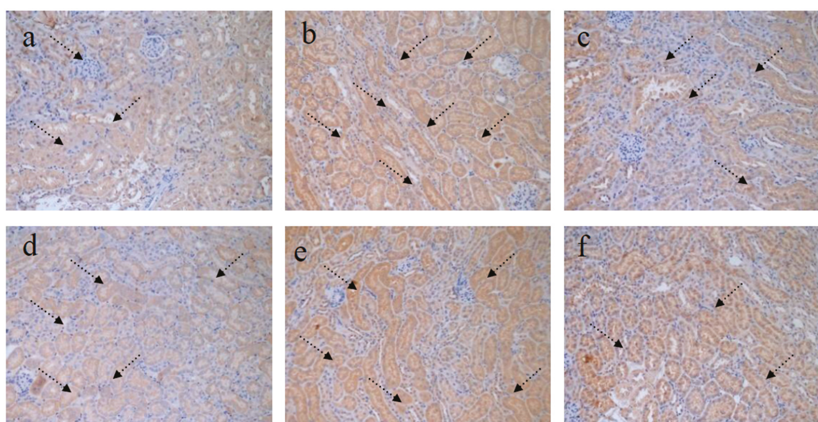


Figure 7. Effect of RPP on the TGF-β1of kidney (TGF-β1 stain, 200×). (**a**) NG (Normal group); (**b**) MG (STZ, Model group); (**c**) PG (STZ, DMBG 10 mg/kg); (**d**) RPP-L(STZ, 50 mg/kg RPP); (**e**) RPP-M (STZ, 100 mg/kg RPP); (**f**) RPP-H (STZ, 200 mg/kg RPP).

5. Conclusions

In this study, T2DMmice model was successfully developed through feeding with low-dose STZ combined with high-fat diet. The effect of RPP on biochemical indicators of serum and liver in mice were determined. Furthermore, histopathology of liver, kidney, and pancreas and immunohistochemistry of kidney were evaluated. The results indicated that RPP effectively reduced the damage in STZ-induced diabetic mice. This study introduced methods and provided data as basis for the potential applications of RPP as pharmaceutical and food ingredient.

Acknowledgments: We gratefully thank the National Natural Science Foundation of China (Grant No. 31360381, 31301456) for the financial support on this research.

Author Contributions: Yongliang Zhuang and Liping Sun conceived and designed the experiments; Liping Sun; Qingyu Ma and Yan Guo completed the model establishment of T2DM; Liping Sun, Qingyu Ma and Yan Guo completed the experiments; Qingyu Ma and Yan Guo assisted in collecting the data and interpreted the results; Yongliang Zhuang and Liping Sun provided the reagents, analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

References

1. Gopal, S.S.; Lakshmi, M.J.; Sharavana, G.; Sathaiah, G.; Sreerama, Y.N.; Baskaran, V. Lactucaxanthin a potential anti-diabetic carotenoid from lettuce (*Lactuca sativa*) inhibits α-amylase and α-glucosidase activity in vitro and in diabetic rats. *Food Funct.* **2017**, *8*, 1124–1131. [CrossRef] [PubMed]

2. Shi, G.J.; Zheng, J.; Wu, J.; Qiao, H.Q.; Chang, Q.; Niu, Y.; Sun, T.; Li, Y.X.; Yu, J.Q. Beneficial effects of Lyciumbarbarum polysaccharide on spermatogenesis by improving antioxidant activity and inhibiting apoptosis in streptozotocin-induced diabetic male mice. *Food Funct.* **2017**, *8*, 1215–1226. [CrossRef] [PubMed]
3. Prince, P.S.M.; Kamalakkannan, N.; Menon, V.P. Antidiabetic and antihyperlipidemic effect of alcoholic Syzigiumcumini seeds in alloxan induced diabetic albino rats. *J. Ethnopharm.* **2004**, *91*, 209–213. [CrossRef] [PubMed]
4. Liu, W.; Wei, Z.; Ma, H.; Cai, A.; Liu, Y.; Sun, J.; DaSilva, N.A.; Johnson, S.L.; Kirschenbaum, L.; Cho, B.P.; et al. Anti-glycation and anti-oxidative effects of a phenolic-enriched maple syrup extract and its protective effects on normal human colon cells. *Food Funct.* **2017**, *8*, 757–766. [CrossRef] [PubMed]
5. Sun, D.; Huang, S.; Cai, S.; Cao, J.; Han, P. Digestion property and synergistic effect on biological activity of purple rice (*Oryza sativa* L.) anthocyanins subjected to a simulated gastrointestinal digestion in vitro. *Food Res. Int.* **2015**, *78*, 114–123. [CrossRef] [PubMed]
6. Alkan, D.; Yemenicioglu, A. Potential application of natural phenolic antimicrobials and edible film technology against bacterial plant pathogens. *Food Hydrocol.* **2016**, *55*, 1–10. [CrossRef]
7. Sun, L.; Guo, Y.; Zhang, Y.; Zhuang, Y. Antioxidant and Anti-tyrosinase Activities of Phenolic Extracts from Rape Bee Pollen and Inhibitory Melanogenesis by cAMP/MITF/TYR Pathway in B16 Mouse Melanoma Cells. *Front. Pharmacol.* **2017**, *8*, 104. [CrossRef] [PubMed]
8. Ma, T.; Sun, X.; Tian, C.; Zheng, Y.; Zheng, C.; Zhan, J. Chemical composition and hepatoprotective effects of polyphenols extracted from the stems and leaves of Sphallerocarpus gracilis. *J. Funct. Foods* **2015**, *18*, 673–683. [CrossRef]
9. Lee, Y.-H.; Kim, J.-H.; Kim, S.H.; Oh, J.Y.; Seo, W.D.; Kim, K.-M.; Jung, J.-C.; Jung, Y.-S. Barley Sprouts Extract Attenuates Alcoholic Fatty Liver Injury in Mice by Reducing Inflammatory Response. *Nutrients* **2016**, *8*, 440. [CrossRef] [PubMed]
10. Jeszka-Skowron, M.; Flaczyk, E.; Jeszka, J.; Krejpcio, Z.; Król, E.; Buchowski, M.S. Mulberry leaf extract intake reduceshyper glycaemia in streptozotocin (STZ)-induced diabetic rats fed high-fat diet. *J. Func. Foods* **2014**, *8*, 9–17. [CrossRef]
11. Cherbal, A.; Kebieche, M.; Yilmaz, E.M.; Aydoğmuş, Z.; Benzaouia, L.; Benguessoum, M.; Benkedidah, M.; Madani, K. Antidiabetic and hypolipidemic activities of Algerian *Pistachia lentiscus* L. leaves extract in alloxan-induced diabetic rats. *S. Afr. J. Bot.* **2017**, *108*, 157–162. [CrossRef]
12. Liu, Z.; Zhai, J.; Han, N.; Yin, J. Assessment of anti-diabetic activity of the aqueous extract of leaves of Astilboidestabularis. *J. Ethnopharm.* **2016**, *194*, 635–641. [CrossRef] [PubMed]
13. Boaventura, B.C.B.; Pietro, P.F.D.; Kleina, G.A.; Stefanuto, A.; de Morais, E.C.; de Andrade, F.; Wazlawik, E.; da Silva, E.L. Antioxidant potential of mate tea (Ilex paraguariensis) in type 2 diabetic mellitus and pre-diabetic individuals. *J. Func. Foods* **2013**, *5*, 1057–1064. [CrossRef]
14. Chung, I.M.; Kim, E.H.; Yeo, M.A.; Kim, S.J.; Seo, M.C.; Moon, H.I. Antidiabetic effects of three Korean sorghum phenolic extracts in normal and streptozotocin-induced diabetic rats. *Food Res. Int.* **2011**, *44*, 127–132. [CrossRef]
15. Vadivel, V.; Biesalski, H.K. Contribution of phenolic compounds to the antioxidant potential and type II diabetes related enzyme inhibition properties of *Pongamia pinnata* L. Pierre seeds. *Process Biochem.* **2011**, *46*, 1973–1980. [CrossRef]
16. Taha, H.; Ary, A.; Paydar, M.; Looi, C.Y.; Wong, W.F.; Murthy, C.R.V.; Noordin, M.I.; Ali, H.M.; Mustafa, A.M.; Hadi, A.H.A. Upregulation of insulin secretion and down regulationof pro-inflammatory cytokines, oxidative stress and hyperglycemiain STZ-nicotinamide-induced type 2 diabetic rats by Pseuduvaria monticola bark extract. *Food Chem. Toxicol.* **2014**, *66*, 295–306. [CrossRef] [PubMed]
17. Sun, L.P.; Zhang, H.L.; Zhuang, Y.L. Preparation of free, soluble conjugate, and insoluble-bound phenolic compounds from peels of rambutan (*Nephelium lappaceum*) and evaluation of antioxidant activities in vitro. *J. Food Sci.* **2012**, *77*, C198–C204. [CrossRef] [PubMed]
18. Zhuang, Y.; Ma, Q.; Guo, Y.; Sun, L. Purification and identification of rambutan (*Nephelium lappaceum*) peel phenolics with evaluation of antioxidant and antiglycation activities in vitro. *Int. J. Food Sci. Technol.* **2017**. [CrossRef]
19. Zhuang, Y.; Ma, Q.; Guo, Y.; Sun, L. Protective effects of rambutan (*Nephelium lappaceum*) peel phenolics on H2O2-induced oxidantive damages in HepG2 cells and D-galactose-induced aging mice. *Food Chem. Toxicol.* **2017**. [CrossRef]

20. Chen, P.; Zhang, Q.; Dang, H.; Liu, X.; Tian, F.; Zhao, J.; Chen, Y.; Zhang, H.; Chen, W. Oral administration of Lactobacillus rhamnosus CCFM0528 improves glucose tolerance and cytokine secretion on high-fat-fed, streptozotocin-induced type 2 diabetic mice. *J. Func. Foods* **2014**, *10*, 318–326. [CrossRef]
21. Priscilla, D.H.; Jayakumar, M.; Thirumurgan, K. Flavanone naringenin: An effective antihyperglycemic and antihyperlipidemic nutraceutical agent on high fat diet fed streptozotocin induced type 2 diabetic rats. *J. Func. Foods* **2015**, *14*, 363–373. [CrossRef]
22. Mehenni, C.; Atmani-kilani, D.; Dumarcay, S.; Perrin, D.; Gérardin, P.; Atmani, D. Hepatoprorective and antidiabetic effects of Pistacia lentiscus leaf and fruit extracts. *J. Food Drug Anal.* **2016**, *24*, 653–669. [CrossRef]
23. Xu, J.; Wang, Y.; Xu, D.S.; Ruan, K.F.; Feng, Y.; Wang, S. Hypoglycemic effects of MDG-1, a polysaccharide derived from Ophiopogon japonicas, in the ob/ob mouse model of type 2 diabetes mellitus. *Int. J. Biol. Macromol.* **2011**, *49*, 657–662. [CrossRef] [PubMed]
24. Li, Y.; Ji, D.; Zhong, S.; Lin, T.; Lv, Z. Hypoglycemic effect of deoxynojirimycin-polysaccharide on high fat diet and streptozotocin-induced diabetic mice via regulation of hepatic glucose metabolism. *Chemico-Bio. Int.* **2015**, *225*, 70–79. [CrossRef] [PubMed]
25. Pan, L.H.; Li, X.F.; Wang, M.N.; Zha, X.Q.; Yang, X.F.; Liu, Z.J.; Luo, Y.B.; Luo, J.P. Comparision of hypoglycemic and antioxidant effects of polysaccharides from four different Dendrobium species. *Int. J. Biol. Macromol.* **2014**, *64*, 420–427. [CrossRef] [PubMed]
26. Irudayaraj, S.S.; Sunil, C.; Duraipandiyan, V.; Ignacimuthu, S. Antidiabetic and antioxidant acticities of *Toddalia asiatica* (L.) Lam. leaves in streptozotoncin induced daibeitc rats. *J. Ethnopharm.* **2012**, *143*, 515–523. [CrossRef] [PubMed]
27. Xia, X.; Yan, J.; Shen, Y.; Tang, K.; Yin, J.; Zhang, Y. Berberine improves glucose metabolism in diabetic rats by inhibition of hepatic gluconeogenesis. *PLoS ONE* **2011**, *6*, e16556. [CrossRef] [PubMed]
28. Wang, K.; Tang, Z.; Wang, J.; Cao, P.; Li, Q.; Shui, W.; Wang, H.; Zheng, Z.; Zhang, Y. Polysaccharide from Angelica sinensis ameliorates high-fat diet and STZ-induced hepatic oxidative stress and inflammation in diabetic mice by activating the Sirt1-AMPK pathway. *J. Nutr. Biochem.* **2017**, *43*, 88–97. [CrossRef] [PubMed]
29. Sun, L.; Zhang, Y.; Zhuang, Y. Antiphotoaging effect and purification of an antioxidant peptide from tilapia (*Oreochromis niloticus*) gelatin peptides. *J. Func. Foods* **2013**, *5*, 154–162. [CrossRef]
30. Arya, A.; Looi, C.Y.; Cheah, S.C.; Mustafa, M.R.; Mohd, M.A. Anti-diabetic effects of Centratherum anthelminticum seeds methanolic fraction on pancreatic cells, β-TC6 and its alleviating role in type 2 diabetic rats. *J. Ethnopharmacol.* **2012**, *144*, 22–32. [CrossRef] [PubMed]
31. Chinchansure, A.A.; Korwar, A.M.; Kulkarni, M.J.; Joshi, S.P. Recent development of plant products with anti-glycation acitvity: A review. *RSC Adv.* **2015**, *5*, 31113–31138. [CrossRef]
32. Pinent, M.; Blay, M.; Bladé, M.C.; Salvadó, M.J.; Arola, L.; Ardévol, A. Grape seedderived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic acitivity in insulin-sensitive cell lines. *Endocrinology* **2004**, *145*, 4985–4990. [CrossRef] [PubMed]
33. Nankar, R.P.; Doble, M. Ellagic acid potentiates insulin sensitising activity of pioglitazone in L6 myotubes. *J. Func. Foods* **2015**, *15*, 1–10. [CrossRef]
34. Sutariya, B.; Saraf, M. Betanin, isolated from fruits of Opuntiaelatior Mill attenuates renal fibrosis in diabetic rats through regulating oxidative stress and TGF-β pathway. *J. Ethnopharmacol.* **2017**, *198*, 432–443. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Effects of Vegetables on Cardiovascular Diseases and Related Mechanisms

Guo-Yi Tang [1], Xiao Meng [1], Ya Li [1], Cai-Ning Zhao [1], Qing Liu [1] and Hua-Bin Li [1,2,*]

[1] Guangdong Provincial Key Laboratory of Food, Nutrition and Health, Department of Nutrition, School of Public Health, Sun Yat-Sen University, Guangzhou 510080, China; tanggy5@mail2.sysu.edu.cn (G.-Y.T.); mengx7@mail2.sysu.edu.cn (X.M.); liya28@mail2.sysu.edu.cn (Y.L.); zhaocn@mail2.sysu.edu.cn (C.-N.Z.); liuq248@mail2.sysu.edu.cn (Q.L.)

[2] South China Sea Bioresource Exploitation and Utilization Collaborative Innovation Center, Sun Yat-Sen University, Guangzhou 510006, China

* Correspondence: lihuabin@mail.sysu.edu.cn; Tel.: +86-20-873-323-91

Received: 10 July 2017; Accepted: 6 August 2017; Published: 10 August 2017

Abstract: Epidemiological studies have shown that vegetable consumption is inversely related to the risk of cardiovascular diseases. Moreover, research has indicated that many vegetables like potatoes, soybeans, sesame, tomatoes, dioscorea, onions, celery, broccoli, lettuce and asparagus showed great potential in preventing and treating cardiovascular diseases, and vitamins, essential elements, dietary fibers, botanic proteins and phytochemicals were bioactive components. The cardioprotective effects of vegetables might involve antioxidation; anti-inflammation; anti-platelet; regulating blood pressure, blood glucose, and lipid profile; attenuating myocardial damage; and modulating relevant enzyme activities, gene expression, and signaling pathways as well as some other biomarkers associated to cardiovascular diseases. In addition, several vegetables and their bioactive components have been proven to protect against cardiovascular diseases in clinical trials. In this review, we analyze and summarize the effects of vegetables on cardiovascular diseases based on epidemiological studies, experimental research, and clinical trials, which are significant to the application of vegetables in prevention and treatment of cardiovascular diseases.

Keywords: cardiovascular disease; vegetable; bioactive component; effect; mechanism

1. Introduction

Cardiovascular diseases (CVDs) have spread worldwide, and their prevalence is increasing dramatically [1,2]. CVDs have become one of the biggest threats to people's health, as reported by the World Health Organization (WHO), and CVDs caused 17.7 million deaths (coronary heart disease (CHD): 7.4 million, stroke: 6.7 million) in 2015, accounting for 31% of all global deaths [3]. Besides high morbidity and mortality, CVDs also lead to serious disabilities and decrease the living standards of patients, creating a huge burden for individuals, families, and countries [4–6]. Therefore, it is urgent and worthwhile to investigate prevention and treatment strategies of CVDs [7].

CVDs are a class of chronic non-infectious diseases related to substantial complicated risk factors such as high blood pressure, hyperlipidemia, diabetes, overweight and obesity, metabolic syndrome, smoking, excessive alcohol consumption, imbalanced diet, and a lack of physical activity [6,8–13]. Efficient strategies can be applied to preventing and treating CVDs by targeting these risk factors, e.g., reducing blood pressure, regulating the blood lipid profile, reducing oxidative stress, modulating inflammatory status, inhibiting thrombosis, and attenuating myocardial damage as well as ameliorating metabolism syndrome [13–19]. Meanwhile, a healthy lifestyle, including a balanced diet, reasonable physical activity, moderate alcohol consumption, and stopping smoking, is beneficial to persons at high risk of CVDs [13,20–23]. Among these methods, establishing and insisting on a

healthy eating pattern would be a substantial, sustainable, and economical choice. As recommended in the 2015–2020 Dietary Guidelines for Americans, people should follow a rational eating pattern with the aim of achieving and maintaining good health and lowering the risk of chronic diseases like CVDs, diabetes, overweight, and obesity throughout all stages of life [24].

It has been proven by epidemiological studies that vegetable consumption is negatively associated with the risks of CVDs [25–30]. Furthermore, evidence from experimental research suggested that many vegetables were effective in preventing CVDs, such as potatoes, soybeans, sesame, tomatoes, dioscorea, onions, celery, broccoli, lettuce, and asparagus [31–35]. Some bioactive components might account for the cardioprotective effects of vegetables, like vitamins, essential elements, dietary fibers, botanic proteins, and phytochemicals [36–41]. The potential mechanisms of action could involve antioxidation; anti-inflammation; anti-platelet; regulating blood glucose, lipid profile, and blood pressure; and attenuating myocardial damage [42–45]. In addition, clinical trials indicated that the consumption of several vegetables was beneficial to cardiovascular health [46–49]. The present review summarizes the effects of vegetables on CVD prevention and treatment, with special attention paid to the mechanisms of action.

2. Epidemiological Studies

Numerous epidemiological studies have indicated that vegetables were inversely associated with CVD incidence and many kinds of vegetables possessed cardioprotective effects, such as tomatoes, potatoes, onions, cereals, and cruciferous vegetables [26–30]. Moreover, a variety of bioactive components in vegetables have been proven to convey health benefits in preventing and treating CVDs, like botanical protein, dietary fiber, vitamins, essential elements, and phytochemicals [27,28,50].

2.1. Cross-Sectional Studies

Several cross-sectional studies evaluated the relationship between vegetable intake and CVD risks [26,51,52]. It was found that total cholesterol (TC), the TC/high density lipoprotein cholesterol (HDL-C) ratio, and hemoglobin A1c were significantly improved in women who consumed more than 10 servings/week of tomato-based food products compared to those consuming fewer than 1.5 servings/week [51]. Specifically, a significant improvement was observed in women with higher consumption compared to those with lower consumption, i.e., TC (5.38 mmol/L vs. 5.51 mmol/L, $p = 0.029$), the TC/HDL-C ratio (4.08 vs. 4.22, $p = 0.046$), and hemoglobin A1c (5.02% vs. 5.13%, $p < 0.001$), and consumers with higher intake were 31% (95% confidence intervals (CI): 6–50%), 40% (95% CI: 13–59%), and 66% (95% CI: 20–86%) less likely to have increased TC ($\geq$6.21 mmol/L), low-density lipoprotein cholesterol (LDL-C) ($\geq$4.14 mmol/L), and hemoglobin A1c ($\geq$6%), respectively. In another study of 4774 Iranian subjects, significant correlations between potato intake and diabetes, high fasting blood sugar level as well as low serum HDL level were observed (odds ratio (OR): 1.38, 95% CI: 1.14–1.67, $p < 0.001$; OR: 1.40, 95% CI: 1.17–1.68, $p < 0.001$; OR: 1.10, 95% CI: 1.01–1.20, $p = 0.02$; respectively) [26]. These results suggested a potential effect of potato consumption on CVDs, as high fasting blood glucose, low serum HDL, and diabetes are recognized as CVD risk factors. Furthermore, it was indicated in a cross-sectional study with 3995 Mediterranean participants at high CVD risk that gazpacho (a Mediterranean vegetable-based cold soup that contains plenty of phytochemicals) intake was negatively correlated with hypertension [52]. It was found that both the systolic and diastolic blood pressure of the participants reduced with means of −1.9 mm Hg (95% CI: −3.4 to −0.6) and −2.6 mm Hg (95%CI: −4.2 to −1.0) and of −1.5 mm Hg (CI: −2.3 to −0.6) and −1.9 mm Hg (95% CI: −2.8 to −1.1), respectively, in moderate (1 to 19 g/day) and high (more than 20 g/day) gazpacho intake categories, compared with the control group. Moreover, the incidence of hypertension was decreased after gazpacho intake of 250 g/week and for high gazpacho intake groups compared with the control group, with OR = 0.85 (95% CI: 0.73–0.99) and OR = 0.73 (95% CI: 0.55–0.98), respectively.

2.2. Case-Control Studies

Similar results were illustrated in case-control studies investigating the association between vegetable intake and CVD risks [27,53–55]. In one study, the relationship between onion intake and acute myocardial infarction (MI) in Italy was analyzed [53]. Compared to the control group, the risks of acute MI for the group consuming less than one portion of onion per week and more than one portion per week significantly decreased (OR = 0.90, 95% CI: 0.69–1.21 and OR = 0.78, 95% CI: 0.56–0.99, respectively). Another case-control study in Korea suggested that consuming vegetables was inversely correlated with stroke risk [27]. It was shown that participants who consumed four to six servings of vegetables per day and more than six servings per day were 32% and 69% less likely, respectively, to suffer from stroke. Researchers also found that intake of vitamin B1, vitamin B2, vitamin B6, niacin, folate, calcium, and potassium was significantly inversely correlated with stroke risk. In addition, the effects of vegetable consumption on the connection between hypertension and the relative telomere length of peripheral leukocytes were measured in a study [54]. On one hand, it was reported that longer age-adjusted relative telomere length was related to higher vegetable consumption ($p = 0.01$). On the other hand, subjects with longer age-adjusted relative telomere length were 30% less likely to suffer from hypertension (OR = 0.70, 95% CI: 0.52–0.96, $p = 0.03$). The significant and negative connection between hypertension-relative telomere length and hypertension was only observed in those with greater (more than 150 g/day) vegetable intake (OR = 0.28, 95% CI: 0.14–0.57, $p < 0.001$), but not in those with lower (less than 50 g/day) vegetable intake ($p = 0.008$). Interestingly, in a study conducted in central Iran, evidence indicated that there was a marginally significant independent association between potato consumption and risk of stroke [55]. If compared with those with the lowest (5.3 ± 0.4 g/day) consumption, subjects with the highest (60.0 ± 6.1 g/day) potato consumption were more likely to have strokes (OR: 1.9, 95% CI: 1.0–3.6).

2.3. Cohort Studies

It has been confirmed by cohort studies that vegetable consumption was inversely relative to CVDs including hypertension, stroke, CHD, and even death [28,30,50]. Vegetables exerted a protective effect on CVD patients. A cohort study in Spain reported that cereal protein and fiber were negatively correlated with hypertension risk [28]. Cereal protein and fiber significantly lowered hypertension risk in participants in the highest quintile of intake compared with those in the lowest (hazard ratio (HR) = 0.5, 95% CI: 0.2–0.9, $p = 0.06$ and HR = 0.6, 95% CI 0.3–1.0, $p = 0.05$, respectively). Researchers also found that the risk reduction was more significant in older individuals compared to the young, in men compared to women, and in obese patients compared to those with a normal body weight. In another study, lycopene consumption was negatively correlated to CVD risks after a nine-year follow-up (HR = 0.83, 95% CI: 0.70–0.98), and to CHD risk after an 11-year follow-up (HR = 0.74, 95% CI: 0.58–0.94) [50]. Moreover, researchers found that vegetable intake could help decrease the risk of total mortality, with HRs (95% CIs) for total mortality across increasing quintiles of intake at 1 (reference), 0.88 (0.79–0.97), 0.88 (0.79–0.98), 0.76 (0.62–0.92), and 0.84 (0.69–1.00) for total vegetables ($p = 0.03$), and 1 (reference), 0.91 (0.84–0.98), 0.88 (0.77–1.00), 0.85 (0.76–0.96), and 0.78 (0.71–0.85) for cruciferous vegetables ($p < 0.0001$) [30]. Therefore, their findings supported the notion that increasing intake of vegetables, especially cruciferous vegetables, might help reduce CVD risk.

However, some studies found no significant inverse connection between vegetable consumption and CVD risks [56,57]. On the one hand, researchers focused on the connection between vegetable flavonoid intake and CVD risk in women in a cohort study, only to find that for both CVDs and important cardiovascular events, there was no significant linear trend across quintiles of vegetable flavonoid consumption ($p = 0.63$ and 0.80, respectively), and neither for the individual flavonol or flavone [56]. According to prospective data from another cohort study, broccoli consumption was not significantly associated with a reduction of CVD risk, and no significant association between broccoli flavonol or flavone consumption and nonfatal MI or fatal CHD risk was observed in U.S. women [57]. On the other hand, harmful components were found in a few kinds of vegetables or badly cooked

ones [58,59]. The findings of a study indicated that habitually high consumption of soybean isoflavones might modestly but significantly increase the risks of ischemic stroke in women [58]. The HRs from the lowest (median intake: 6.0 mg/day) to the highest (median intake: 53.6 mg/day) quintiles were 1.00, 1.05, 1.10, 1.11, and 1.24, respectively (95% CI: 1.08–1.42, $p = 0.002$). In another study, researchers found that the HRs for subjects consuming four or more servings per week were 1.11 (95% CI: 0.96–1.28, $p = 0.05$) for baked, boiled, or mashed potatoes, 1.17 (95% CI: 1.07–1.27, $p = 0.001$) for French fries, and 0.97 (95% CI: 0.87–1.08, $p = 0.98$) for potato chips, compared with those consuming less than one serving per month [59]. These results indicated that a higher intake of badly cooked potato might increase the risks of developing hypertension independently and prospectively.

2.4. Other Epidemiological Studies

Besides the studies mentioned above, there are some epidemiological studies aiming to find an association between vegetable consumption and CVDs. Results from these epidemiological studies are also promising, as presented in Table 1.

Table 1. Other vegetables associated with CVDs.

Vegetables	Subjects	Effects	References
Vegetables with carotenoids	U.S. male physicians aged 40–84 years (n = 15,220)	Lowered the risks of CHD	[60]
Onion quercetin		Incorporated into the atherosclerotic region, acted as a complementary antioxidant	[61]
soybean isoflavones	Chinese adults (n = 572)	Lowered serum TAG, carotid artery intima-media thickness, increased HDL-C	[62]
soybean foods, isoflavones	(Meta-analysis)	reduced ischemic heart disease, lowered blood LDL-C, improved endothelial function, slowed the progression of subclinical atherosclerosis	[63]
Green leafy vegetables	(Meta-analysis)	Reduced incidence of CVDs significantly (15.8%)	[29]
Nitrate-containing vegetables	Non-hypertensive subjects aged 20–70 years (n = 1546)	Had a protective effect against development of hypertension	[64]

In summary, evidence from most epidemiological studies suggested the significant negative relationship between vegetable consumption and the risks of CVDs. These vegetables specifically included tomato, potato, onion, cereal and cruciferous vegetables. Several kinds of components, like botanic protein, dietary fiber, vitamins (vitamin B1, vitamin B2, niacin and folate), essential elements (calcium and potassium) and phytochemicals (lycopene), might contribute to the cardioprotective effects of vegetables. However, in some studies, there were no observed significant inverse association between CVDs risks and intake of broccoli and vegetable flavonols. In some other studies, consuming potato, particularly badly cooked potato, could even increase the risk of CVD.

3. Experimental Research

3.1. Potatoes

People all over the word consume a large amount of potatoes per year. Potatoes have been found to benefit the cardiovascular system, thus they are worth investigating for the treatment and prevention of CVDs [42,43]. Researchers investigated the potential effects of a CA Mey (Hypoxidaceae) corm (African potato) aqueous extract (APE) on the cardiovascular system in experimental animal paradigms [42]. Firstly,

APE (25–400 mg/mL) exhibited negative inotropic effects on guinea pig isolated electrically driven left atrial muscle preparations and negative chronotropic effects on spontaneously beating right ones, respectively, significantly ($p < 0.05$–0.001) and concentration-dependently. Secondly, APE concentration-dependently reduced or abolished the positive inotropic and chronotropic reactions of strips of atrial muscle from guinea pig induced by noradrenaline (1–100 μM) and calcium (Ca^{2+}, 5–40 mM), which were not modified by exogenous administration of atropine ($7.5 \times 10^{-7} - 2.5 \times 10^{-6}$ M) to the bath fluid. Thirdly, APE also caused a reduction or cessation of the rhythmic, spontaneous, myogenic contractions of portal veins in rats, significantly ($p < 0.05$–0.001) and concentration-dependently. Furthermore, APE reduced the systemic arterial blood pressure as well as heart rates of hypertensive rats, significantly ($p < 0.05$–0.001) and dose-dependently. Taken together, APE might be a natural candidate for cardiac dysfunction and essential hypertension remedy. In another study, cholesterol and triglyceride (TG) levels in plasma (−30%, $p < 0.0001$ and −36%, $p < 0.05$, respectively) and cholesterol levels in the liver (−42%, $p < 0.0001$) were significantly reduced in rats after three weeks of a potato-enriched diet [43]. Antioxidant status was also improved due to the intake of potato; additionally, thiobarbituric acid reactive substances (TBARS) levels in the heart were lowered and the vitamin E/TG (VE/TG) ratio in plasma was improved. These effects indicated that consumption of cooked potato could be a way of preventing CVDs. However, when researchers investigated the effects of soluble fiber extracted from potato pulp on risk factors for diabetes and CVDs in Goto–Kakizaki rats, no difference in hematological parameters was found; only the postprandial plasma TG concentration of rats was reduced, significantly but modestly [65]. These results might lead to a conclusion that plasma cholesterol or glycemic response could not be reduced by increased fermentation and production of propionate with diet-soluble fiber.

3.2. Soybeans

Soybeans are a common vegetable that can be used to extract oil and make soy milk. Polyphenols, mainly including phenolic acid and flavonoids like flavones and flavonols, are among the most important bioactive components extracted from soybeans. It was reported that phenolic acid mainly contributed to the antioxidant capacities of many natural products [66–71]. Many researchers suggested that polyphenols possessed biological effects like antioxidation and anti-inflammation, which in turn provided cardiovascular protection [37,44,45,72–75]. In an in vitro study, phenolic-rich extracts from soybeans were found to inhibit the activities of α-amylase, α-glucosidase, and angiotensin-I converting enzyme (ACE), which are key enzymes linked to diabetes and hypertension [44]. Thus, researchers came to the conclusion that soybeans have health-promoting effects including anti-diabetes and anti-hypertension. Another study investigated the effects of saponin (2-phenyl-benzopyrane), a soybean flavonoid, on glucose tolerance and risk factors for atherosclerosis [45]. In saponin-treated animals, the LDL-C/TG ratio was increased, and TG, very low-density lipoprotein cholesterol (VLDL-C), lipid hydroperoxide, and TC/HDL-C ratio were decreased. However, no effects were found on glucose tolerance, LDL-C, superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the experimental groups. These observations indicated that saponin from soybeans might improve the serum lipid profile due to direct antioxidant activity.

It was also reported that soybeans contain considerable phytoestrogens, like isoflavones (mainly genistein and daidzein) and lignans, which are safe and natural estrogen receptor modulator alternatives to hormone therapy and possess antioxidant and cardioprotective effects [31,32,76]. Researchers analyzed the functional and anatomopathological effects of soybean extract and isoflavone on post-MI [76]. It was found that in the soybean extract group, a protective effect was observed 30 days after the MI. In another study, the cardioprotective effects of genistein from soybean extract on isoproterenol-treated H9c2 cardiomyoblast cells were investigated [31]. Results indicated that genistein administration could downregulate the expression of mitochondrial pro-apoptotic proteins such as Bad, caspase-3, caspase-8, and caspase-9 in H9c2 cells. Additionally, several survival proteins were expressed in H9c2 cells, including phosphor (p)-Akt, p-Bad, and p-Erk1/2. Moreover, researchers reported that genistein exerted cardioprotective effects partially due to the regulation of Erk1/2, Akt,

and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) proteins by inhibiting related pathways. It was also pointed out that genistein from soybeans not only reversed preexisting severe pulmonary hypertension but also prevented its progression into heart failure (HF) [32]. When genistein and daidzein were administered, significant neuroprotective effects and antioxidant activities were observed both in vitro and in vivo in ischemia/reperfusion (I/R)-like conditions [77]. Moreover, the effects of genistein from soybeans on blood pressure were evaluated in fructose-induced hypertensive rats [78]. Results showed that genistein administration could lower blood pressure and restore ACE, protein kinase C-β II, and nitric oxide (NO) synthase (NOS) expression.

Soybean protein is a well-known botanical protein that is regarded as a kind of complete protein, highly valuable in promoting health [79,80]. The cardioprotective effects of soybean protein have been proven by evaluating the association between dietary protein source, protein level, and serum lipid profile in male rats [79]. It was found that the total serum TG level was significantly lowered after long-term intake of soybean protein, indicating the possibility of reducing the risks of atherosclerosis. It was also reported that soybean protein possessed cardioprotective effects, partially by improving serum lipids via modifying the expression of sterol regulatory element-binding protein-2 and its downstream genes (hydroxymethylglutaryl-coenzyme A reductase and LDL receptor), and increasing the antioxidant activities of SOD and catalase [80].

It was reported that soybean products could be enhanced in nutritional value after fermentation [81]. For instance, doenjang was more effective at preventing diet-induced visceral fat accumulation than non-fermented soybeans in rats, by stimulating carnitine palmitoyltransferase-1 activity and suppressing fatty acid synthase activity, possibly due to the higher content of aglycone isoflavones [82]. Additionally, it was evaluated that regular intake of miso soup, a Japanese soybean paste, could alleviate salt-induced sympathoexcitation in mice with chronic pressure overload via inhibiting the hypothalamic MR–AT1R pathway [83]. Moreover, the effects of probiotic-fermented genetically modified (GM) soybean milk on hypercholesterolemia in hamsters were explored [84]. The observations suggested that serum total TG level decreased significantly ($p < 0.05$) after treatment with four kinds of soy milk (GM or non-GM; with or without probiotic fermentation), compared to the control group in a diet with high cholesterol. In addition, there was a significant difference between the GM and non-GM soy milk groups ($p > 0.05$) in total TG levels. Furthermore, the GM soy milk was found to reduce the risk of developing atherosclerosis by alleviating oxidative stress and diminishing atherosclerotic plaque formation in the aorta.

There are some other bioactive components in soybeans, such as unsaponifiables and oligosaccharides, which are beneficial to the cardiovascular system [85,86]. The protective effects of soybean unsaponifiables on the prefrontal cortex after global brain I/R injury in rats were investigated [85]. The results indicated that malondialdehyde (MDA) and tumor necrosis factor-α (TNF-α) levels, as well as the number of apoptotic neurons, were significantly decreased, while SOD activities were significantly increased, suggesting that soybean unsaponifiables had antioxidant and neuroprotective effects. In addition, the protective effects of soybean oligosaccharides on heart function against myocardium I/R injury were assessed in rats [86]. MDA level was upregulated, while antioxidant enzyme activities and the expression of p-JAK2 and p-STAT3 proteins were increased in the soybean-oligosaccharide-treated group. When rats were fed with soybean oligosaccharides, the cardiac contractile function was significantly recovered, the infarct size was reduced, and creatine kinase, aspartate transaminase, and lactate dehydrogenase activities were decreased as well.

3.3. Sesame

It has been demonstrated that extracts of sesame possessed strong antioxidant, anti-atherogenic, anti-thrombotic, and anti-hypertensive activity; thus, regularly consuming sesame whole grains or purified bioactive components would offer effective protection against CVDs [33–35]. In a study, chemical and biological model systems were used to access the free radical scavenging capacity and anti-atherogenic activity of *Sesamum indicum* seed extracts [33]. By Fe^{3+}/ferricyanide complex and

ferric reducing antioxidant power assays, it was reported that any dose (25–1000 μg/mL) of aqueous and ethanolic extracts significantly scavenged the NO, superoxide, 1-diphenyl-2-picrylhydrazyl, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)1, and hydroxyl radicals. In biological models, metal-induced lipid peroxidation in mitochondrial fractions, human serum, and LDL oxidation models was inhibited by both extracts. Moreover, in a lipoprotein kinetics study, the lag phase time was significantly ($p < 0.05$) increased by both extracts, while the oxidation rate as well as the conjugated dienes production was reduced. In another study, the anti-hypertensive effects of ACE inhibitory peptides from a sesame protein hydrolysate in spontaneously hypertensive rats (SHRs) were investigated [34]. The systolic blood pressure in SHRs was significantly and temporarily lowered by sesame peptide powder at 1 and 10 mg/kg, which might be due to ACE inhibitory activity. Moreover, the ACE activity was competitively inhibited by the representative peptides (Leu-Val-Tyr, Leu-Gln-Pro, and Leu-Lys-Tyr) isolated from sesame peptide powder at Ki = 0.92 μM, 0.50 μM, and 0.48 μM, respectively. According to the content ratio in sesame peptide powder, it was evident that a reconstituted sesame peptide mixture of Leu-Ser-Ala, Leu-Gln-Pro, Leu-Lys-Tyr, Ile-Val-Tyr, Val-Ile-Tyr, Leu-Val-Tyr, and Met-Leu-Pro-Ala-Tyr exhibited a strong anti-hypertensive effect on SHRs at doses of 3.63 and 36.3 μg/kg, which were responsible for more than 70% of the corresponding dosage for hypotensive effects induced by the sesame peptide powder. Furthermore, researchers focused on the anti-thrombotic effects of sesame, and found that Col/Chichibu/Maruteru-2/1995 and T016 varieties of sesame exhibited significant anti-thrombotic activity, while 00037803 was pro-thrombotic [35]. It was also observed that sesamol was the most effective component, followed by sesamolin and sesamin, which showed significant acute anti-thrombotic effects.

Although it was the fat-soluble constituents in the sesame that were thought to benefit the cardiovascular system, some studies demonstrated that defatted sesame seed extracts (DSSE) also possessed protective effects [87,88]. In a study, researchers evaluated the positive effects of DSSE using ischemia models [87]. It was found that DSSE (0.1–10 μg/mL) significantly blocked cell death and prevented lipid peroxidation induced by oxygen–glucose deprivation followed by reoxygenation. It was also evident that brain infarct volume was reduced in a dose-dependent manner, while sensory and motor function were improved by DSSE (30, 100, and 300 mg/kg, orally) administrated 0 h and 2 h after the onset of ischemia. Therefore, it could be concluded that DSSE might be effective in ischemia models due to the antioxidant activity. In another study, researchers investigated whether the neuroprotective effects of DSSE were related to brain edema [88]. The results showed that water content leakage was reduced by DSSE (30, 100, and 300 mg/kg, orally), but not Evans blue leakage. The Aquaporin 4 expression was inhibited by DSSE at 4 h but not at 24 h after ischemia. No effect on matrix metalloproteinase expressions and activities was observed. Herein, DSSE might be effective on brain edema due to the regulation of Aquaporin 4 during the acute phase of ischemia.

3.4. Tomatoes

Tomatoes were thought to have a considerable protective role in CVD; in particular, their bioactive component, lycopene, was found to exhibit significant antioxidant, anti-hypertensive, hypolipidemic, and anti-atherogenic effects in vivo and in vitro [36,39]. In a study, it was showed that the increase in serum MB-isoenzyme of creatine phosphokinase (CPK-MB) was prevented and cardiac cell injury was ameliorated by lycopene (1.7 and 3.5 mg/kg, intraparietally) and tomato extract (1.2 and 2.4 g/kg, intraparietally), respectively [36]. These results suggested that lycopene and tomato extract inhibited the cardiotoxicity induced by doxorubicin and could be used in combination with doxorubicin to alleviate the organ injury induced by free radicals. In another study, researchers investigated the effects of tomato extracts and carotenoids, like lycopene and lutein, on physiological function and NF-κB signaling in endothelial cells [39]. All carotenoids could cause a significant improvement in primary endothelial function, which was related to increase NO and decreased endothelin release. In addition, carotenoids effectively attenuated inflammatory NF-κB signaling, including reducing the adhesion of leukocytes induced by TNF-α, expression of adhesion molecules (AM) like inter-cellular

adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), nuclear translocation of NF-κB components, and reverting the inhibitor of κB ubiquitination. Additionally, carotenoids played a role in inhibiting NF-κB activation in transfected endothelial cells. Moreover, lutein combined with oleoresin synergistically precluded leukocytes' adhesion.

Sapogenol, another major bioactive component in tomatoes, exhibited anti-atherogenic activities endowing tomatoes with cardioprotective effects [89,90]. It was reported that esculeogenin A, a new tomato sapogenol, ameliorated hyperlipidemia and atherosclerosis in ApoE-deficient mice via restraining cholesterol acyl-transferase [89]. Esculeogenin A markedly blocked the accumulation of cholesterol ester induced by acetylated LDL in human monocyte-derived macrophages and Chinese hamster ovary cells, dose-dependently. In addition, esculeogenin A prevented the expression of acyl-coenzyme A: cholesterol acyl-transferase (ACAT)-1 protein, and suppressed the activities of both ACAT-1 and ACAT-2. The levels of serum cholesterol, TG, LDL-C, as well as the proportion of atherosclerotic lesions in ApoE-deficient mice were significantly decreased by oral administration of esculeoside A, without any detectable side effects. In a similar study, tomatidine, a tomato sapogenol, was reported to significantly suppress the activity of cholesterol acyl-transferase and led to the reduction of atherogenesis [90].

In addition, the n-hexane extract of tomato exerted a protective effect against adrenaline-induced MI in rats [91]. The levels of MDA in heart and aspartate aminotransferase in serum were both significantly lowered in adrenaline-treated rats given a pre-treatment of tomato extract (1 mg/kg, 2 mg/kg) and vitamin E (50 mg/kg), which also significantly blocked myocardial necrosis. It could be concluded that the n-hexane extract of tomato possessed an antioxidative potential that might in turn prevent MI induced by catecholamine. Additionally, the anti-hypertensive effects of a tomato cultivar (DG03-9) rich in gamma-aminobutyric acid (GABA) were investigated in SHRs [92]. DG03-9 caused a significant reduction in systolic blood pressure with both single and chronic administration compared to the control. Moreover, researchers found that DG03-9 elicited a higher anti-hypertensive effect than the commonly consumed cultivar (Momotaro) did, and GABA exhibited a similar effect to DG03-9 in a comparable dose. Furthermore, it was reported that consuming cooked tomato sauce could preserve coronary endothelial function; improve HDL, apolipoprotein A-I, and apolipoprotein J protein profile; enhance endothelial NOS transcription and activation; and reduce DNA damage in the coronary arteries in dyslipidemic animals [93]. These bioactivities were responsible for the beneficial effects of cooked tomato sauce, i.e., lowing lipid peroxidation, increasing HDL antioxidant potential, and preventing diet-induced impairment of receptor-operated and non-receptor-operated endothelial-dependent coronary vasodilation.

3.5. Dioscorea

Dioscorea is a common vegetable widely used as traditional Chinese medicine, and contains a variety of bioactive components, like saponins, diosgenin, and flavonoids; it has been demonstrated that saponins have anti-thrombotic activity [40,41]. In a study, the total steroidal saponins derived from *Dioscorea zingiberensis* rhizomes blocked platelet aggregation and thrombosis dose-dependently, leading to prolonged activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT) in rats and prolonged bleeding time and clotting time in mice, suggesting ability to decrease CVD risk [40]. In another study, researchers evaluated the anti-thrombotic effects of four kinds of diosgenyl saponins [41]. The observations indicated that diosgenyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside, a novel disaccharide saponin, exhibited outstanding efficiency in prolonging bleeding time. Moreover, it could significantly and dose-dependently block platelet aggregation, prolong APTT, and inhibit factor VIII activities in rats. Taken together, a conclusion could be drawn that diosgenyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside had considerable anti-thrombotic activity. Moreover, the beneficial effects of total saponins exacted from three medicinal species of dioscorea, *Dioscorea nipponica* Makino, *Dioscorea panthaica* Prain et Burkill, and *Dioscorea zingiberensis*, against

isoprenaline-induced myocardial ischemia were further investigated [94]. It was found that the total saponins from the three dioscorea significantly reduced activities of creatine kinase, lactate dehydrogenase, and aspartate aminotransferase; lowered the concentration of MDA; and increased activities of total SOD, catalase, GPx, and total antioxidant capacity, which was comparable between these three dioscorea. Additionally, heart tissue from total saponins groups revealed less severe histological damage. These results might partially explain why total saponins possess a cardioprotective efficacy for myocardial ischemia. Furthermore, saponins exhibited a potent neuroprotective property in attenuating severe injury induced by transient focal cerebral I/R, and the mechanism included anti-inflammatory and anti-apoptotic action [95]. It was reported that saponins markedly decreased neurological deficit scores, cerebral infarct volume, and brain edema in rats. Additionally, saponins increased neuron survival (Nissl bodies) and decreased caspase-3 in the hippocampal cornu Ammons 1 and cortex hemisphere of the ipsilateral ischemia. Moreover, pre-administration of saponins significantly decreased the inflammatory cytokines in serum induced by the middle cerebral artery occlusion, and markedly inhibited the downregulating anti-apoptotic Bcl-2 and upregulating proapoptotic Bax proteins.

It was reported that dioscorea, and its bioactive compound diosgenin in particular, exerted anti-thrombosis activity, possibly via promoting the anti-coagulation function and blocking platelet aggregation [96,97]. In a study, it was found that platelet aggregation, thrombosis and APTT, TT, and PT in rats were dose-dependently inhibited by diosgenin, while the bleeding time and clotting time were dose-dependently prolonged in mice [96]. It could be concluded that diosgenin extracted from *Dioscorea zingiberensis* possessed anti-thrombosis activities with a potential for CVD treatment. In another study, diosgenin was observed to alleviate cardiotoxicity induced by doxorubicin in mice [97]. In the heart tissue, diosgenin recovered the reduced activities of antioxidant enzymes, involving SOD and GPx. In addition, diosgenin significantly lowered the serum levels of cardiotoxicity markers, cardiac levels of TBARS and reactive oxygen species (ROS), caspase-3 activation, mitochondrial dysfunction, and the expression of NF-κB. Moreover, diosgenin increased the cardiac levels of cyclic guanosine monophosphate by modulating phosphodiesterase-5 activity and attenuating myocardial fibrosis. Meanwhile, it was confirmed that regulating protein kinase A and p38 could mediate the health benefits of diosgenin. These results implied that diosgenin possessed antioxidant and anti-apoptotic activities, as well as cyclic guanosine monophosphate modifying effects, which in turn protected the heart from cardiotoxicity induced by doxorubicin.

There are some other studies focusing on the beneficial effects of dioscorea on cardiovascular protection as well. More promisingly, other bioactive compounds contained in dioscorea have been identified, which might protect against MI and atherosclerosis [98,99]. In a study, results suggested that the flavonoid-rich portion of *Dioscorea bulbifera* Linn. could attenuate lipid peroxidation due to the capacity to scavenge free radicals and modulate energy-producing mitochondrial enzymes, suggesting a cardioprotective effect on isoproterenol-induced MI [98]. In another study, researchers arrived at the conclusion that an extract of Chinese yam, rich in β-sitosterol and ethyl linoleate, had the capability to prevent atherosclerosis, thus it could be a candidate for functional foods. It was reported that such extracts could inhibit the expression of inflammatory mediators, including TNF, NO, and inducible NOS, and the development of atherosclerotic lesions [99]. In addition, several studies also suggested the cardioprotective effects of dioscorea, of which the bioactive compounds might not have been identified [100,101]. In a study, it was confirmed that dioscorea rhizome exhibited antioxidative and anti-atherogenic effects on hyperlipidemic rabbits, suggesting that supplementation with dioscorea rhizome might be a possible way to reduce oxidative stress and attenuate atherosclerosis [100]. In another study, *Dioscorea opposita* Thunb. was found to exhibit anti-hypertensive effects on hypertensive rats through inhibiting the endothelin-converting enzymes as well as antioxidant activity [101]. After treatment, *Dioscorea opposita* Thunb. caused significant reductions in mean blood pressure, plasma endothelin and MDA concentration, plasma angiotensin-II activity, left ventricular hypertrophy, and cardiac mass index, while increasing the plasma SOD activity.

3.6. Onions

Onions are a commonly consumed vegetable all over the world, and contain bioactive components like phytochemicals. Onion extracts exhibited potent anti-atherogenic effects that were related to a variety of bioactivities [102,103]. In a study, onion (*Allium cepa* L.) extracts as well as the bioactive components quercetin and catechin were observed to enhance paraoxonase 1 activity and radical scavenging activity, which in turn prevented LDL oxidation and lipid peroxidation in male Wistar rats subjected to oxidative stress caused by mercuric chloride [102]. In another study, [103] onion extract was found to lessen atherosclerotic lesions, increase endogenous aortic hydrogen sulfide (H_2S) production, and decrease plasma adrenomedulin content, aortic adrenomedulin content, aortic calcitonin receptor-like receptor, and receptor activity-modifying protein 1/2 mRNAs. Additionally, plasma GPx level, SOD activity, plasma endothelial NOS activity, and NO content were increased, while MDA and inflammatory response were reduced by onion extract. All of these effects made onions a potential candidate for anti-atherogenic therapy.

Some experimental studies have suggested that onions have anti-thrombotic effects via platelet inhibitory response and inhibiting mitogen-activated protein kinase (MAPK) activation. Therefore, onion intake might have a capacity for preventing platelet-mediated CVDs [104–106]. In a study, results showed that onion could inhibit thrombosis induced by platelets in dogs [104]. It was demonstrated that periodic platelet-mediated thrombus formation followed by embolization caused a reduction in cyclic flow. However, in five dogs, 0.09 ± 0.01 mL/kg onion juice administered intravenously attenuated cyclic flow reductions within 20 min, followed by a $60 \pm 14\%$ ($p = 0.002$) reduction in collagen-induced ex vivo whole blood platelet aggregation. In addition, in six dogs given 2.0 g/kg onion homogenate intragastrically, cyclic flow reductions were lessened within 2.5–3 h in five of the dogs, accompanied by a $44 \pm 24\%$ ($p = 0.04$) reduction in ex vivo aggregation. Moreover, as measured by thrombosis/thrombolysis models in rodents in another study, a variety of onion cultivars exhibited natural anti-thrombotic effects [105]. First of all, researchers confirmed that Toyohira exerted marked anti-thrombotic activities as well as anti-platelet effects accompanied by thrombolytic activity. Meanwhile, Super Kita Momiji, 2935A, and K83211 exhibited only thrombolytic activity. Furthermore, researchers found no significant association between quercetin concentration and anti-thrombotic activity. Interestingly, the anti-thrombotic effects of quercetin-rich onion peel extracts (OPE) on arteries in rats were stated in another study [106]. The OPE markedly reduced blood TG and glucose without affecting blood cholesterol levels. In addition, in vivo arterial thrombosis was significantly abolished in groups fed with 2 mg and 10 mg OPE. Additionally, thrombin-induced expression of tissue factor in human umbilical vein endothelial cells, a coagulation initiator, was greatly diminished by the OPE. Furthermore, extracellular signal-regulated kinase (ESRK) and c-Jun N-terminal kinase (CJNK) signaling pathways activated by thrombin treatment were blocked by pre-treatment with OPE.

Onions were also found to have anti-hypertensive effects in some other experiments [107,108]. For instance, dietary onion decreased the TBARS in plasma in N(G)-nitro-L-arginine methyl ester (L-NAME)—induced-hypertensive rats and stroke-prone SHRs [107]. In addition, onions improved the nitrate/nitrite (products of NO) excreted in urine and the NOS activities in the kidneys in stroke-prone SHRs, but not in L-NAME- induced-hypertensive rats. These results might in part explain the mechanisms by which onion exerted an anti-hypertensive effect on these hypertensive rats. In addition, the anti-hypertensive effects of onion were observed with different mechanisms [108]. OPE was demonstrated to concentration-dependently reduce the aorta contractions induced by KCl or phenylephrine ($p < 0.001$). Moreover, the OPE activity could not be attenuated by removing aorta endothelium, or the inhibition of NO, cGMP and prostaglandin synthesis induced by L-NAME (100 μM), methylene blue (10 μM) and indomethacin (10 μM), respectively. In addition, the relaxation in phenylephrine-precontracted aorta mediated by OPE was not abolished by atropine, which blocked the acetylcholine-induced relaxation. Furthermore, after three weeks' intervention with OPE, a reduction of blood pressure was observed in the hypertensive rats fed with fructose ($p < 0.001$).

3.7. Other Vegetables

Besides the vegetables investigated above, there are others that have beneficial effects on the cardiovascular system. Evidence from experimental research has suggested cardioprotective effects and mechanisms (Table 2).

In summary, numerous experimental studies have indicated that vegetable consumption is potentially beneficial in preventing and treating CVD. As demonstrated, vegetables like potatoes, soybeans, sesame, tomatoes, dioscorea, and onions possess cardioprotective effects, for which a variety of bioactive components including vitamins, essential elements, dietary fiber, botanical proteins, and phytochemicals are responsible. In addition, the cardioprotective effects might include antioxidation, anti-inflammation, anti-platelet, lowering blood pressure, modifying lipid metabolism, regulating blood glucose, improving endothelial function, and attenuating myocardial damage (Figure 1). Moreover, the mechanisms of action might involve modulating related enzyme activity, gene expression, and signaling pathways as well as some other biomarkers associated with CVD risk (Table 3).

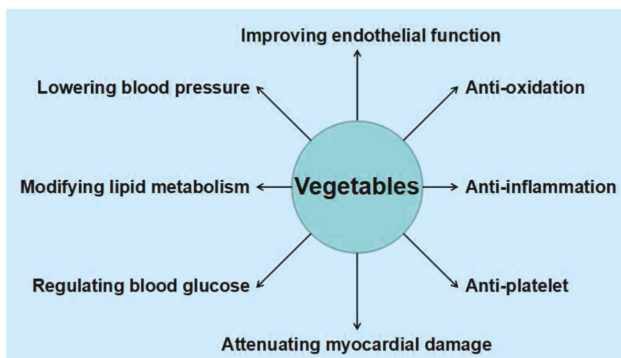


Figure 1. The cardioprotective effects of vegetables.

Table 2. Other vegetables and their effects on CVDs.

Vegetables	Subjects	Effects and Mechanisms	References
Apium graveolens (seed)	Rats	Decreased blood pressure, increased heart rate	[109]
Celery (seed)	RAW264.7 macrophages	Lessened lipid droplets and TC content, decreased secretion of inflammatory cytokine TNF-α and interleukin (IL)-6, promoted cell viability, inhibited apoptosis, suppressed NF-κB, p65 and notch1 protein expressions	[110]
Apium graveolens (leaf)	Sprague Dawley rats	Decreased systolic blood pressure, cholesterol, TG, LDL and VLDL	[111]
Asparagus officinalis	SHRs	Lowered systolic blood pressure, urinary protein excretion/creatinine excretion ratio, creatinine clearance and ACE activity	[112]
Lettuce	Rats	Decreased LDL/HDL ratio and liver cholesterol levels, increased fecal total steroid excretion, depressed apparent absorption of dietary cholesterol, improved VE/TG ratio in plasma, limited lipid peroxidation in heart	[113]
Collard greens	SHRs	Modulated liver fatty acid composition, protected against elevations in atherogenic fatty acids	[114]
Brassica oleracea L.	In vitro thrombolytic model	Showed clot lysis activity	[115]
Rape (seed)	SHRs	Inhibited ACE, dilated mesenteric artery	[116]
Rape (seed)	SHRs	Inhibited ACE and renin activities, lowered blood pressure	[117]
Rape (seed)	SHRs	Reduced surface hydrophobicity, scavenged oxygen radicals, inhibited ACE, lowered blood pressure	[118]
Spinach	Balb/c mice	Decreased catalase, increased SOD activities, protected against doxorubicin-induced heart injury	[119]
Spinach	SHRs	Exerted anti-hypertensive activity	[120]
Spinach (leaf)	SHRs	Inhibited ACE, exerted anti-hypertensive activity	[121]
Pumpkin	In vitro	Antioxidant, inhibited α-glucosidase and ACE, anti-diabetic- and anti-hypertension	[122]
Daucus carota	In vitro, normotensive anesthetized rats	Lowered arterial blood pressure, inhibited spontaneously beating guinea pig atria and K^+ -induced contractions of rabbit aorta	[123]
Lyophilized carrot	C57BL/6J mice	Increased total neutral sterols fecal excretion, increased antioxidant status and VE/TG ratio, lowered lipemia, regulated cholesterol metabolism	[124]
Carrot	In vitro, mice	Anti-thrombosis	[125]
Broccoli	stroke-prone SHRs	Attenuated oxidative stress, hypertension and inflammation	[126]

Table 2. *Cont.*

Vegetables	Subjects	Effects and Mechanisms	References
Broccoli	Rats	Protected mammalian hearts, activated survival proteins, improved post-ischemic ventricular function and pro-caspase 3 activities and redox cycling of thioredoxins, reduced myocardial infarct size, cardiomyocyte apoptosis and cytochrome c release	[127]
Broccoli	Rats	Protected against myocardial oxidative damage and cell death during I/R, inhibited markers of necrosis and apoptosis, decreased oxidative stress	[128]
Broccoli	Rats	Improved post-ischemic ventricular function, reduced MI and cardiomyocyte apoptosis	[129]
Corn	SHRs, in vitro	Inhibited ACE, lowered systolic blood pressure	[130]
Corn	In vitro	Antioxidant, inhibited a-glucosidase and ACE, anti-diabetic- and anti-hypertension	[122]
Purple corn	SHRs	Decreased blood pressure and heart rate	[131]
Maize	Wistar rats	Reduced infarct size, increased myocardial glutathione levels, modulated cardiac antioxidant defenses	[132]
Pea	Rats	Reduced MDA, tissue calcium concentration, myeloperoxidase and apoptosis indicator caspase-3, protected hearts from I/R injury	[133]
Latyrus cicera	Rats	Hindered free radical-mediated tissue injury, endothelial dysfunction and leukocyte recruitment, protected against splanchnic artery I/R-induced splanchnic injury	[134]
Pea	In vitro	Inhibited α-amylase and α-glucosidase and ACE	[135]
Pea	Weanling Han:SPRD-cy rats	Lowered serum creatinine and renal chemokine receptor 2 level	[136]
Pea	Rats	lowered plasma TC concentrations, affected cellular cholesterol homeostasis	[137]

Table 3. The mechanisms involved in the cardioprotective effects of vegetables.

Cardioprotective Effects	Mechanisms
Lower blood pressure	Inhibit ACE activity and hypothalamic MR-ATIR pathway, alleviate sympathoexcitation; improve protein kinase C-β II activity; modify relative telomere length of peripheral leucocyte, increase NOS expression; inhibit Ca^{2+} influx and K^{+} -induced contractions.
Regulate lipid metabolism	Decrease TC, TG, TAG, VLDL-C, TC/HDL-C ratio and atherosclerotic plaque formation, increase LDL-C/TG and VE/TG ratio; inhibit fatty acid synthase and ACAT activity, modulate energy producing mitochondrial enzymes; modify expression of ACAT and sterol regulatory element-binding protein-2 and its downstream genes.
Antioxidant	Scavenge free radicals (NO, superoxide, hydroxyl, TBARS); increase endogenous aortic H_2S production; improve SOD, catalase, GPx, carnitine palmitoyltransferase-1 and paraoxonase 1 activity.
Improve endothelial function	Decrease endothelin and artery intima-media thickness, increase NO, improve apolipoprotein A-I and apolipoprotein J protein profile; inhibit endothelin-converting enzyme; diminish DNA damage.
Anti-inflammatory	Attenuate TNF-α induced leukocytes adhesion; reduce NF-κB, IL-6; inhibit expression of AM.
Anti-platelet	Prolong APTT, TT, PT, bleeding time and clotting time; inhibit MAPK, ESRK, factor VIII activities and c-Jun N-terminal kinase signaling pathways.
Attenuate myocardial damage	Decrease MDA, water content leakage and infarct size, increase cyclic guanosine monophosphate; inhibit creatine kinase, aspartate transaminase, lactate dehydrogenase and CPKMB activity, modulate protein kinase A, p38, and phosphodiesterase-5 activity; inhibit Bad, Bax, caspase-8, caspase-9, and caspase-3 and aquaporin 4 expression, increase phosphor (p)-Akt, p-Bad, p-Erk1/2, Bcl-2, p-JAK2 and p-STAT3.
Regulate blood glucose	Inhibit α-amylase and α-glucosidase activity; improve hemoglobin A1c and high fasting blood sugar level.

4. Clinical Trials

4.1. Whole Soybeans and Soy Milk

In addition to the findings in the abovementioned studies that soybeans possess a variety of bioactivities to prevent CVD, the results of many clinical trials have supported that soybean consumption might be a way to lower CVD risk and maintain cardiovascular health [46–49]. Soybeana had an effect on biomarkers of CVDs in elderly women with metabolic syndrome [46]. Compared to mean changes from baseline with the control group, LDL-C, VLDL-C, and apolipoprotein B100 levels were significantly improved in the whole soybean (35 g/day) intake group ($p < 0.05$), while fewer, significant improvements were observed in these variables in the textured soybean protein (35 g/day) group ($p < 0.001$). For apolipoprotein A-I, similar results were observed in the treatment groups ($p < 0.01$), in which serum TC was significantly decreased ($p < 0.005$). In all, soybeans and textured soybean protein could improve the lipid profile, but the former caused more considerable improvements than the latter. Moreover, consumption of soybean foods was found to improve the lipid profile in patients with hyperlipidemia [47]. According to the data measured in the separation of the group into equol producers ($n = 30$) and non-producers ($n = 55$), similar changes from baseline in LDL-C were observed (−9.3 ± 2.5% and −11.1 ± 1.6%, respectively, $p = 0.834$), with preservation of HDL-C and apolipoprotein A-I only in equol producers compared with changes in non-producers (HDL-C: +0.9 ± 2.7% compared with −4.3 ± 1.1%, $p = 0.006$; apolipoprotein A-I: −1.0 ± 1.1% compared with −4.7 ± 1.0%, $p = 0.011$). Moreover, soy milk consumption significantly reduced systolic blood pressure in type 2 diabetic patients with nephropathy when compared to cow's milk consumption (percent change: −4.50% vs. 5.89%, $p = 0.03$) [48]. Additionally, intake of soy milk significantly reduced

serum TG (percent change: −15.22% vs. 2.37%, $p = 0.02$), though these effects were not significant after adjustment for carbohydrate intake. Furthermore, data derived from another study indicated that the use of soybean products in comprehensive early rehabilitation therapy of patients with macrofocal MI significantly reduced the risk of arrhythmia [49].

4.2. Soybean Protein

Soybean protein could modestly improve the serum lipid profile as well as other risk factors related to CVD, as has been affirmed in a study involved 90 moderately hypercholesterolemic Chinese adults [138]. In a randomized controlled trial (RCT), soybean protein supplementation gave rise to a significant mean net change (95% CI) in plasma E-selectin of −3.93 ng/mL (−7.05 to −0.81 ng/mL, $p = 0.014$) compared with milk protein, and in plasma leptin of −2089.8 pg/mL (−3689.3 to −490.3 pg/mL, $p = 0.011$) compared with carbohydrate [139]. These observations indicated that soybean protein supplementation could reduce E-selectin and leptin levels in plasma. However, intake of either cow's milk or a soy protein beverage for eight weeks did not alter soluble cell AM concentrations in pre-hypertensive or stage 1 hypertensive individuals, suggesting that neither beverage diminished atherosclerotic CVD risk in mildly hypertensive individuals by improving circulating cell AM concentrations [140].

4.3. Soybean Isoflavones

Soybean isoflavones, especially genistein and daidzein, are common phytoestrogens recognized as selective estrogen receptor modulators that possess cardioprotective effects in vitro and in vivo, but there is a lack of promising outcomes in clinical trials. In a six-month RCT, purified daidzein did not exhibit significant effects on body weight, body mass index, waist and hip circumferences, waist to hip ratio, body fat percentage, fat mass, and free fat mass in equol-producing postmenopausal women with prehypertension [141]. In the same study, it was found that purified daidzein had no significant effect on blood pressure and vascular function [142]. However, in the above two studies urinary isoflavones suggested good compliance of the patients with the interventions.

4.4. Combination of Soybean Isoflavones and Soybean Protein

Results from clinical trials also indicated that a combination of isoflavones and soybean protein might not be an effective intervention to prevent CVD [143,144]. In a RCT, isoflavone soybean protein (ISP) supplementation did not result in a significant reduction of subclinical atherosclerosis progression in postmenopausal women [143]. While subgroup analysis indicated that ISP supplementation could reduce subclinical atherosclerosis in healthy young women (median age: 53 years) less than five years postmenopausal who were at low risk for CVDs. In a double-blind randomized, placebo-controlled trial conducted among 180 postmenopausal Chinese women, soybean protein combined with isoflavones at the provided dosage (15 g soybean protein, 100 mg isoflavones) had no significant effect on measured cardiovascular risk factors, including serum HDL-C, LDL-C, TC, TG, and highly sensitive C-reactive protein [144].

4.5. Other Vegetables and Their Bioactive Components

Other vegetables and their bioactive components were also studied, such as sesame, tomatoes, broccoli, and onions, among which some were found to have properties promising for CVD prevention and treatment (Table 4).

Table 4. Relationship between other vegetables and CVD.

Vegetables	Subjects	Effects	References
Sesame	Overweight or obese men and women (*n* = 33)	No improvement in markers of CVD risk	[145]
Tomato	Patients with grade-1 hypertension (*n* = 31)	Decreased blood pressure and TBARS level	[146]
Tomato	Healthy women (*n* = 18)	Improved serum antioxidant status, decreased vascular AM 1	[147]
Tomato	Healthy subjects (*n* = 40)	Decrease plasma TC, TG and several cellular and plasma inflammatory biomarkers, increase plasma HDL-C and IL-10	[148]
Tomato	Healthy middle-aged volunteers (*n* = 225)	No change in inflammatory markers, insulin resistance and sensitivity, lipid concentrations and arterial stiffness	[149]
Broccoli	Hypertensive individuals (*n* = 40)	No significant change in blood pressure and endothelial function measured by flow mediated dilation	[150]
Broccoli	Healthy Caucasian volunteers (*n* = 24)	Increased the urinary concentrations of sulforaphane metabolites and vitamin C, decreased the urinary concentrations of tetranor-PGEM, 11β-PGF2α and 11-dehydro-TXB2	[151]
Onion	Healthy men (*n* = 23)	Improved postprandial but not fasting flow-mediated vasodilation; did not alter systemic and forearm hemodynamics	[152]
Onion	Overweight-to-obese patients (*n* = 70)	Decreased 24 h, daytime and night-time systolic blood pressure in hypertensives; did not affect vasoactive biomarkers	[153]

In addition, healthy dietary patterns characterized by a high content of vegetables were important to reduce CVD risk [154,155]—for instance, the recommended Dietary Approach to Stop Hypertension (DASH) and the Mediterranean diet. The DASH diet suggests consumption of vegetables, fruits, and low-fat dairy products, and was found to result in a significant improvement of cardiovascular risk factors including BP, TC, and LDL, and cause a risk reduction for CVD incidence and mortality [156,157]. The Mediterranean dietary pattern is characterized by a high content of vegetables, as well as fruits and whole grains, and has been reported to decrease the incidence and mortality of CVDs, like CHD, MI, and stroke [158–160].

In summary, clinical trials showed that some specific vegetables had advantages in CVD prevention and treatment. Whole soybeans and their components (like soy protein) possessed potent cardioprotective effects. Moreover, some other vegetables, such as sesame, tomatoes, broccoli, and onions, were beneficial to CVD patients to some degree. On the other hand, no significant change in some biomarkers in subjects consuming tomato, broccoli, and soybean isoflavones was observed in some studies, so further clinical trials regarding the cardioprotective effects of vegetables are warranted. Furthermore, it is favorable to promote a healthy dietary pattern containing a high content of vegetable to reduce CVD risk.

5. Conclusions

The results from many epidemiological studies support the hypothesis that vegetable consumption is inversely correlated to the risk of CVDs. Moreover, numerous studies have suggested that many vegetables could be taken into consideration as candidates for CVD prevention and treatment, such as potatoes, soybeans, sesame, tomatoes, dioscorea, onions, celery, broccoli, lettuce, and

asparagus, which contain varieties of bioactive components, including vitamins, essential elements, dietary fibers, botanical proteins, and phytochemicals. The cardioprotective effects of vegetables might include antioxidation, anti-inflammation, anti-platelet, lowering blood pressure, modifying lipid metabolism, regulating blood glucose, improving endothelial function, attenuating myocardial damage, modulating related enzyme activities, gene expressions and signaling pathways, as well as some other biomarkers associated with CVD risk. Furthermore, the cardioprotective effects of vegetables have also been observed in some clinical trials, though evidence was limited. Thus, consuming vegetables could help maintain cardiovascular health, and could be used as a substantial, sustainable, and economical strategy. In the future, more vegetables should be evaluated as to whether they have protective effects on the cardiovascular system, and bioactive components should be isolated and identified. Underlying mechanisms of action are also worth investigating. Meanwhile, more clinical trials should be conducted in this field.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (No. 81372976), a Key Project of the Guangdong Provincial Science and Technology Program (No. 2014B020205002), and the Hundred-Talents Scheme of Sun Yat-Sen University.

Author Contributions: Guo-Yi Tang and Hua-Bin Li conceived this paper; Guo-Yi Tang, Xiao Meng, Ya Li, Cai-Ning Zhao, and Qing Liu wrote this paper; Hua-Bin Li revised the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Liu, M.B.; Wang, W.; Zhou, M.G. Trend analysis on the mortality of cardiovascular diseases from 2004 to 2010 in China. *Chin. J. Epidemiol.* **2013**, *34*, 985–988.
2. Celermajer, D.S.; Chow, C.K.; Marijon, E.; Anstey, N.M.; Woo, K.S. Cardiovascular disease in the developing world: Prevalences, patterns, and the potential of early disease detection. *J. Am. Coll. Cardiol.* **2012**, *60*, 1207–1216. [CrossRef] [PubMed]
3. WHO. Cardiovascular Diseases (CVDs). Available online: http://www.who.int/cardiovascular_diseases/en/ (accessed on 13 March 2017).
4. Yazdanyar, A.; Newman, A.B. The burden of cardiovascular disease in the elderly: Morbidity, mortality, and costs. *Clin. Geriatr. Med.* **2009**, *25*, 563–577. [CrossRef] [PubMed]
5. Zaina, S.; Lund, G. Epigenetics: A tool to understand diet-related cardiovascular risk? *J. Nutrigenet. Nutrigenom.* **2011**, *4*, 261–274. [CrossRef] [PubMed]
6. Praveen, P.A.; Roy, A.; Prabhakaran, D. Cardiovascular disease risk factors: A childhood perspective. *Indian J. Pediatr.* **2013**, *80* (Suppl. 1), 3–12. [CrossRef] [PubMed]
7. Walker, J. Reducing cardiovascular disease risk: Cholesterol and diet. *Nurs. Stand.* **2013**, *28*, 48–55. [CrossRef] [PubMed]
8. Gomez, D.L.C.A.; Gomez, M.M.; Ferrando, V.P.; Barianca, O.M.; Abiatua, B.I.; Posada, D.L.P.M. Prevalence of cardiovascular risk factors in a cohort of affected by the toxic oil syndrome. *Med. Clin.* **2003**, *121*, 405–407. [CrossRef]
9. Tam, C.F.; Nguyen, L.; Pe, S.S.; Hajyan, K.; Kevork, S.; Davis, R.; Poon, G.; Lew, P. The effects of age, gender, obesity, health habits, and vegetable consumption frequency on hypertension in elderly Chinese Americans. *Nutr. Res.* **2005**, *25*, 31–43. [CrossRef]
10. Wens, I.; Dalgas, U.; Stenager, E.; Eijnde, B.O. Risk factors related to cardiovascular diseases and the metabolic syndrome in multiple sclerosis—A systematic review. *Mult. Scler.* **2013**, *19*, 1556–1564. [CrossRef] [PubMed]
11. Anthony, D.; George, P.; Eaton, C.B. Cardiac risk factors: Environmental, sociodemographic, and behavioral cardiovascular risk factors. *FP Essent.* **2014**, *421*, 16–20. [PubMed]
12. Li, S.; Gan, L.Q.; Li, S.K.; Zheng, J.C.; Xu, D.P.; Li, H.B. Effects of herbal infusions, tea and carbonated beverages on alcohol dehydrogenase and aldehyde dehydrogenase activity. *Food Funct.* **2014**, *5*, 42–49. [CrossRef] [PubMed]
13. Zhou, Y.; Zheng, J.; Li, S.; Zhou, T.; Zhang, P.; Li, H.B. Alcoholic beverage consumption and chronic diseases. *Int. J. Environ. Res. Public Health* **2016**, *13*, 522. [CrossRef] [PubMed]

14. Kones, R.; Rumana, U. Prevention of cardiovascular disease: Updating the immensity of the challenge and the role of risk factors. *Hosp. Pract. (1995)* **2014**, *42*, 92–100. [CrossRef] [PubMed]
15. Singh, S.; Kullo, I.J.; Pardi, D.S.; Loftus, E.J. Epidemiology, risk factors and management of cardiovascular diseases in IBD. *Nat. Rev. Gastroenterol. Hepatol.* **2015**, *12*, 26–35. [CrossRef] [PubMed]
16. Uthman, O.A.; Hartley, L.; Rees, K.; Taylor, F.; Ebrahim, S.; Clarke, A. Multiple risk factor interventions for primary prevention of cardiovascular disease in low- and middle-income countries. *Cochrane Database Syst. Rev.* **2015**. [CrossRef]
17. Gan, R.Y.; Xu, X.R.; Song, F.L.; Kuang, L.; Li, H.B. Antioxidant activity and total phenolic content of medicinal plants associated with prevention and treatment of cardiovascular and cerebrovascular diseases. *J. Med. Plants Res.* **2010**, *4*, 2438–2444.
18. Zhang, J.J.; Li, Y.; Zhou, T.; Xu, D.P.; Zhang, P.; Li, S.; Li, H.B. Bioactivities and health benefits of mushrooms mainly from China. *Molecules* **2016**, *21*, 938. [CrossRef] [PubMed]
19. Mozaffarian, D. Dietary and policy priorities for cardiovascular disease, diabetes, and obesity: A comprehensive review. *Circulation* **2016**, *133*, 187–225. [CrossRef] [PubMed]
20. Kwok, C.S.; Umar, S.; Myint, P.K.; Mamas, M.A.; Loke, Y.K. Vegetarian diet, seventh day Adventists and risk of cardiovascular mortality: A systematic review and meta-analysis. *Int. J. Cardiol.* **2014**, *176*, 680–686. [CrossRef] [PubMed]
21. Naja, F.; Nasreddine, L.; Itani, L.; Dimassi, H.; Sibai, A.M.; Hwalla, N. Dietary patterns in cardiovascular diseases prevention and management: Review of the evidence and recommendations for primary care physicians in Lebanon. *J. Med. Liban.* **2014**, *62*, 92–99. [CrossRef] [PubMed]
22. Dutton, G.R.; Laitner, M.H.; Perri, M.G. Lifestyle interventions for cardiovascular disease risk reduction: A systematic review of the effects of diet composition, food provision, and treatment modality on weight loss. *Curr. Atheroscler. Rep.* **2014**, *16*. [CrossRef] [PubMed]
23. Funtikova, A.N.; Navarro, E.; Bawaked, R.A.; Fito, M.; Schroder, H. Impact of diet on cardiometabolic health in children and adolescents. *Nutr. J.* **2015**, *14*. [CrossRef] [PubMed]
24. USDA & USHHS. 2015–2020 Dietary Guidelines for Americans. Available online: http://health.gov/dietaryguidelines/2015/guidelines/executive-summary/ (access on 7 January 2015).
25. Wang, X.; Ouyang, Y.; Liu, J.; Zhu, M.; Zhao, G.; Bao, W.; Hu, F.B. Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: Systematic review and dose-response meta-analysis of prospective cohort studies. *Br. Med. J.* **2014**, *349*. [CrossRef] [PubMed]
26. Khosravi-Boroujeni, H.; Mohammadifard, N.; Sarrafzadegan, N.; Sajjadi, F.; Maghroun, M.; Khosravi, A.; Alikhasi, H.; Rafieian, M.; Azadbakht, L. Potato consumption and cardiovascular disease risk factors among Iranian population. *Int. J. Food Sci. Nutr.* **2012**, *63*, 913–920. [CrossRef] [PubMed]
27. Park, Y. Intakes of vegetables and related nutrients such as vitamin B complex, potassium, and calcium, are negatively correlated with risk of stroke in Korea. *Nutr. Res. Pract.* **2010**, *4*, 303–310. [CrossRef] [PubMed]
28. Alonso, A.; Beunza, J.J.; Bes-Rastrollo, M.; Pajares, R.M.; Martinez-Gonzalez, M.A. Vegetable protein and fiber from cereal are inversely associated with the risk of hypertension in a Spanish cohort. *Arch. Med. Res.* **2006**, *37*, 778–786. [CrossRef] [PubMed]
29. Pollock, R.L. The effect of green leafy and cruciferous vegetable intake on the incidence of cardiovascular disease: A meta-analysis. *JRSM Cardiovasc. Dis.* **2016**, *5*. [CrossRef] [PubMed]
30. Zhang, X.L.; Shu, X.O.; Xiang, Y.B.; Yang, G.; Li, H.L.; Gao, J.; Cai, H.; Gao, Y.T.; Zheng, W. Cruciferous vegetable consumption is associated with a reduced risk of total and cardiovascular disease mortality. *Am. J. Clin. Nutr.* **2011**, *94*, 240–246. [CrossRef] [PubMed]
31. Hu, W.S.; Lin, Y.M.; Ho, T.J.; Chen, R.J.; Li, Y.H.; Tsai, F.J.; Tsai, C.H.; Day, C.H.; Chen, T.S.; Huang, C.Y. Genistein suppresses the isoproterenol-treated H9c2 cardiomyoblast cell apoptosis associated with P-38, Erk1/2, JNK, and NFkappaB signaling protein activation. *Am. J. Chin. Med.* **2013**, *41*, 1125–1136. [CrossRef] [PubMed]
32. Matori, H.; Umar, S.; Nadadur, R.D.; Sharma, S.; Partow-Navid, R.; Afkhami, M.; Amjedi, M.; Eghbali, M. Genistein, a soy phytoestrogen, reverses severe pulmonary hypertension and prevents right heart failure in rats. *Hypertension* **2012**, *60*, 425–430. [CrossRef] [PubMed]
33. Visavadiya, N.P.; Soni, B.; Dalwadi, N. Free radical scavenging and antiatherogenic activities of *Sesamum indicum* seed extracts in chemical and biological model systems. *Food Chem. Toxicol.* **2009**, *47*, 2507–2515. [CrossRef] [PubMed]

34. Nakano, D.; Ogura, K.; Miyakoshi, M.; Ishii, F.; Kawanishi, H.; Kurumazuka, D.; Kwak, C.J.; Ikemura, K.; Takaoka, M.; Moriguchi, S.; et al. Antihypertensive effect of angiotensin I-converting enzyme inhibitory peptides from a sesame protein hydrolysate in spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1118–1126. [CrossRef] [PubMed]
35. Kinugasa, C.; Naemura, A.; Hyodo, K.; Nakai, Y.; Katsuta, M.; Yamamoto, J. Experimental antithrombotic effects of sesame seed whole grains and extracts. *Blood Coagul. Fibrinolysis* **2011**, *22*, 526–531. [CrossRef] [PubMed]
36. Karimi, G.; Ramezani, M.; Abdi, A. Protective effects of lycopene and tomato extract against doxorubicin-induced cardiotoxicity. *Phytother. Res.* **2005**, *19*, 912–914. [CrossRef] [PubMed]
37. Zhang, Y.; Gan, R.; Li, S.; Zhou, Y.; Li, A.; Xu, D.; Li, H. Antioxidant phytochemicals for the prevention and treatment of chronic diseases. *Molecules* **2015**, *20*, 21138–21156. [CrossRef] [PubMed]
38. Deng, G.F.; Lin, X.; Xu, X.R.; Gao, L.L.; Xie, J.F.; Li, H.B. Antioxidant capacities and total phenolic contents of 56 vegetables. *J. Funct. Foods* **2013**, *5*, 260–266. [CrossRef]
39. Armoza, A.; Haim, Y.; Basiri, A.; Wolak, T.; Paran, E. Tomato extract and the carotenoids lycopene and lutein improve endothelial function and attenuate inflammatory NF-kappa B signaling in endothelial cells. *J. Hypertens.* **2013**, *31*, 521–529. [CrossRef] [PubMed]
40. Li, H.; Huang, W.; Wen, Y.; Gong, G.; Zhao, Q.; Yu, G. Anti-thrombotic activity and chemical characterization of steroidal saponins from *Dioscorea zingiberensis* C.H. Wright. *Fitoterapia* **2010**, *81*, 1147–1156. [CrossRef] [PubMed]
41. Zhang, R.; Huang, B.; Du, D.; Guo, X.; Xin, G.; Xing, Z.; Liang, Y.; Chen, Y.; Chen, Q.; He, Y.; et al. Anti-thrombosis effect of diosgenyl saponins in vitro and in vivo. *Steroids* **2013**, *78*, 1064–1070. [CrossRef] [PubMed]
42. Ojewole, J.A.; Kamadyaapa, D.R.; Musabayane, C.T. Some in vitro and in vivo cardiovascular effects of *Hypoxis hemerocallidea* Fisch & CA Mey (Hypoxidaceae) corm (African potato) aqueous extract in experimental animal models. *Cardiovasc. J. S. Afr.* **2006**, *17*, 166–171. [PubMed]
43. Robert, L.; Narcy, A.; Rock, E.; Demigne, C.; Mazur, A.; Remesy, C. Entire potato consumption improves lipid metabolism and antioxidant status in cholesterol-fed rat. *Eur. J. Nutr.* **2006**, *45*, 267–274. [CrossRef] [PubMed]
44. Ademiluyi, A.O.; Oboh, G. Soybean phenolic-rich extracts inhibit key-enzymes linked to type 2 diabetes (alpha-amylase and alpha-glucosidase) and hypertension (angiotensin I converting enzyme) in vitro. *Exp. Toxicol. Pathol.* **2013**, *65*, 305–309. [CrossRef] [PubMed]
45. Rodrigues, H.G.; Diniz, Y.S.; Faine, L.A.; Galhardi, C.M.; Burneiko, R.C.; Almeida, J.A.; Ribas, B.O.; Novelli, E. Antioxidant effect of saponin: Potential action of a soybean flavonoid on glucose tolerance and risk factors for atherosclerosis. *Int. J. Food Sci. Nutr.* **2005**, *56*, 79–85. [CrossRef] [PubMed]
46. Bakhtiary, A.; Yassin, Z.; Hanachi, P.; Rahmat, A.; Ahmad, Z.; Jalali, F. Effects of soy on metabolic biomarkers of cardiovascular disease in elderly women with metabolic syndrome. *Arch. Iran. Med.* **2012**, *15*, 462–468. [PubMed]
47. Wong, J.M.; Kendall, C.W.; Marchie, A.; Liu, Z.; Vidgen, E.; Holmes, C.; Jackson, C.J.; Josse, R.G.; Pencharz, P.B.; Rao, A.V.; et al. Equol status and blood lipid profile in hyperlipidemia after consumption of diets containing soy foods. *Am. J. Clin. Nutr.* **2012**, *95*, 564–571. [CrossRef] [PubMed]
48. Miraghajani, M.S.; Najafabadi, M.M.; Surkan, P.J.; Esmaillzadeh, A.; Mirlohi, M.; Azadbakht, L. Soy milk consumption and blood pressure among type 2 diabetic patients with nephropathy. *J. Ren. Nutr.* **2013**, *23*, 277–282. [CrossRef] [PubMed]
49. Siniavskii, I.; Kraisman, V.A.; Suleimenova, Z. Using of a specialized fermented soy milk product on the basis of soybeans in cardiology practice. *Vopr. Pitan.* **2013**, *82*, 51–57. [PubMed]
50. Jacques, P.F.; Lyass, A.; Massaro, J.M.; Vasan, R.S.; D'Agostino, R.S. Relationship of lycopene intake and consumption of tomato products to incident CVD. *Br. J. Nutr.* **2013**, *110*, 545–551. [CrossRef] [PubMed]
51. Sesso, H.D.; Wang, L.; Ridker, P.M.; Buring, J.E. Tomato-based food products are related to clinically modest improvements in selected coronary biomarkers in women. *J. Nutr.* **2012**, *142*, 326–333. [CrossRef] [PubMed]
52. Medina-Remon, A.; Vallverdu-Queralt, A.; Arranz, S.; Ros, E.; Martinez-Gonzalez, M.A.; Sacanella, E.; Covas, M.I.; Corella, D.; Salas-Salvado, J.; Gomez-Gracia, E.; et al. Gazpacho consumption is associated with lower blood pressure and reduced hypertension in a high cardiovascular risk cohort. Cross-sectional study of the PREDIMED trial. *Nutr. Metab. Cardiovasc. Dis.* **2013**, *23*, 944–952. [CrossRef] [PubMed]

53. Galeone, C.; Tavani, A.; Pelucchi, C.; Negri, E.; La Vecchia, C. Allium vegetable intake and risk of acute myocardial infarction in Italy. *Eur. J. Nutr.* **2009**, *48*, 120–123. [CrossRef] [PubMed]
54. Lian, F.; Wang, J.; Huang, X.; Wu, Y.; Cao, Y.; Tan, X.; Xu, X.; Hong, Y.; Yang, L.; Gao, X. Effect of vegetable consumption on the association between peripheral leucocyte telomere length and hypertension: A case-control study. *Br. Med. J. Open* **2015**, *5*. [CrossRef] [PubMed]
55. Khosravi-Boroujeni, H.; Saadatnia, M.; Shakeri, F.; Keshteli, A.H.; Esmaillzadeh, A. A case-control study on potato consumption and risk of stroke in central Iran. *Arch. Iran. Med.* **2013**, *16*, 172–176. [PubMed]
56. Sesso, H.D.; Gaziano, J.M.; Liu, S.; Buring, J.E. Flavonoid intake and the risk of cardiovascular disease in women. *Am. J. Clin. Nutr.* **2003**, *77*, 1400–1408. [PubMed]
57. Lin, J.; Rexrode, K.M.; Hu, F.; Albert, C.M.; Chae, C.U.; Rimm, E.B.; Stampfer, M.J.; Manson, J.E. Dietary intakes of flavonols and flavones and coronary heart disease in US women. *Am. J. Epidemiol.* **2007**, *165*, 1305–1313. [CrossRef] [PubMed]
58. Yu, D.; Shu, X.O.; Li, H.; Yang, G.; Cai, Q.; Xiang, Y.B.; Ji, B.T.; Franke, A.A.; Gao, Y.T.; Zheng, W.; et al. Dietary isoflavones, urinary isoflavonoids, and risk of ischemic stroke in women. *Am. J. Clin. Nutr.* **2015**, *102*, 680–686. [CrossRef] [PubMed]
59. Borgi, L.; Rimm, E.B.; Willett, W.C.; Forman, J.P. Potato intake and incidence of hypertension: Results from three prospective US cohort studies. *Br. Med. J.* **2016**, *353*. [CrossRef] [PubMed]
60. Liu, S.M.; Lee, I.M.; Ajani, U.; Cole, S.R.; Buring, J.E.; Manson, J.E. Intake of vegetables rich in carotenoids and risk of coronary heart disease in men: The Physicians' Health Study. *Int. J. Epidemiol.* **2001**, *30*, 130–135. [CrossRef] [PubMed]
61. Terao, J.; Kawai, Y.; Murcita, K. Vegetable flavonoids and cardiovascular disease. *Asia Pac. J. Clin. Nutr.* **2008**, *171*, 291–293.
62. Cai, Y.; Guo, K.; Chen, C.; Wang, P.; Zhang, B.; Zhou, Q.; Mei, F.; Su, Y. Soya isoflavone consumption in relation to carotid intima-media thickness in Chinese equol excretors aged 40–65 years. *Br. J. Nutr.* **2012**, *108*, 1698–1704. [CrossRef] [PubMed]
63. Messina, M. Soy foods, isoflavones, and the health of postmenopausal women. *Am. J. Clin. Nutr.* **2014**, *100* (Suppl. 1), 423–430. [CrossRef] [PubMed]
64. Golzarand, M.; Bahadoran, Z.; Mirmiran, P.; Zadeh-Vakili, A.; Azizi, F. Consumption of nitrate-containing vegetables is inversely associated with hypertension in adults: A prospective investigation from the Tehran lipid and glucose study. *J. Nephrol.* **2016**, *29*, 377–384. [CrossRef] [PubMed]
65. Laerke, H.N.; Meyer, A.S.; Kaack, K.V.; Larsen, T. Soluble fiber extracted from potato pulp is highly fermentable but has no effect on risk markers of diabetes and cardiovascular disease in Goto-Kakizaki rats. *Nutr. Res.* **2007**, *27*, 152–160. [CrossRef]
66. Guo, Y.J.; Deng, G.F.; Xu, X.R.; Wu, S.; Li, S.; Xia, E.Q.; Li, F.; Chen, F.; Ling, W.H.; Li, H.B. Antioxidant capacities, phenolic compounds and polysaccharide contents of 49 edible macro-fungi. *Food Funct.* **2012**, *3*, 1195–1205. [CrossRef] [PubMed]
67. Fu, L.; Xu, B.T.; Gan, R.Y.; Zhang, Y.; Xu, X.R.; Xia, E.Q.; Li, H.B. Total phenolic contents and antioxidant capacities of herbal and tea infusions. *Int. J. Mol. Sci.* **2011**, *12*, 2112–2124. [CrossRef] [PubMed]
68. Fu, L.; Xu, B.T.; Xu, X.R.; Qin, X.S.; Gan, R.Y.; Li, H.B. Antioxidant capacities and total phenolic contents of 56 wild fruits from South China. *Molecules* **2010**, *15*, 8602–8617. [CrossRef] [PubMed]
69. Song, F.L.; Gan, R.Y.; Zhang, Y.; Xiao, Q.; Kuang, L.; Li, H.B. Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. *Int. J. Mol. Sci.* **2010**, *11*, 2362–2372. [CrossRef] [PubMed]
70. Xia, E.Q.; Deng, G.F.; Guo, Y.J.; Li, H.B. Biological activities of polyphenols from grapes. *Int. J. Mol. Sci.* **2010**, *11*, 622–646. [CrossRef] [PubMed]
71. Li, H.B.; Wong, C.C.; Cheng, K.W.; Chen, F. Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants. *LWT-Food Sci. Technol.* **2008**, *41*, 385–390. [CrossRef]
72. Li, A.N.; Li, S.; Zhang, Y.J.; Xu, X.R.; Chen, Y.M.; Li, H.B. Resources and biological activities of natural polyphenols. *Nutrients* **2014**, *6*, 6020–6047. [CrossRef] [PubMed]
73. Li, S.; Li, S.K.; Gan, R.Y.; Song, F.L.; Kuang, L.; Li, H.B. Antioxidant capacities and total phenolic contents of infusions from 223 medicinal plants. *Ind. Crop. Prod.* **2013**, *51*, 289–298. [CrossRef]
74. Deng, G.F.; Xu, X.R.; Zhang, Y.; Li, D.; Gan, R.Y.; Li, H.B. Phenolic compounds and bioactivities of pigmented rice. *Crit. Rev. Food Sci. Nutr.* **2013**, *53*, 296–306. [CrossRef] [PubMed]

75. Fu, L.; Xu, B.T.; Xu, X.R.; Gan, R.Y.; Zhang, Y.; Xia, E.Q.; Li, H.B. Antioxidant capacities and total phenolic contents of 62 fruits. *Food Chem.* **2011**, *129*, 345–350. [CrossRef]
76. Miguez, A.C.; Francisco, J.C.; Barberato, S.H.; Simeoni, R.; Precoma, D.; Do, A.V.; Rodrigues, E.; Olandoski, M.; de Noronha, L.; Greca, F.H.; et al. The functional effect of soybean extract and isolated isoflavone on myocardial infarction and ventricular dysfunction: The soybean extract on myocardial infarction. *J. Nutr. Biochem.* **2012**, *23*, 1740–1748. [CrossRef] [PubMed]
77. Valeri, A.; Fiorenzani, P.; Rossi, R.; Aloisi, A.M.; Valoti, M.; Pessina, F. The soy phytoestrogens genistein and daidzein as neuroprotective agents against anoxia-glucopenia and reperfusion damage in rat urinary bladder. *Pharmacol. Res.* **2012**, *66*, 309–316. [CrossRef] [PubMed]
78. Palanisamy, N.; Venkataraman, A.C. Beneficial effect of genistein on lowering blood pressure and kidney toxicity in fructose-fed hypertensive rats. *Br. J. Nutr.* **2013**, *109*, 1806–1812. [CrossRef] [PubMed]
79. Luo, J.; Sun, Z.; Yan, L.; Pan, L.; Yin, S. Effect of different dietary protein source and protein level on serum lipids profile in rats. *Health Res.* **2012**, *41*, 449–452.
80. Marsh, T.G.; Straub, R.K.; Villalobos, F.; Hong, M.Y. Soy protein supports cardiovascular health by downregulating hydroxymethylglutaryl-coenzyme a reductase and sterol regulatory element-binding protein-2 and increasing antioxidant enzyme activity in rats with dextran sodium sulfate-induced mild systemic inflammation. *Nutr. Res.* **2011**, *31*, 922–928. [PubMed]
81. Cai, D.; Liu, M.; Wei, X.; Li, X.; Wang, Q.; Nomura, C.T.; Chen, S. Use of bacillus amyloliquefaciens HZ-12 for High-Level production of the blood glucose lowering compound, 1-Deoxynojirimycin (DNJ), and nutraceutical enriched soybeans via fermentation. *Appl. Biochem. Biotechnol.* **2017**, *181*, 1108–1122. [CrossRef] [PubMed]
82. Kwak, C.S.; Park, S.C.; Song, K.Y. Doenjang, a fermented soybean paste, decreased visceral fat accumulation and adipocyte size in rats fed with high fat diet more effectively than nonfermented soybeans. *J. Med. Food* **2012**, *15*, 1–9. [CrossRef] [PubMed]
83. Ito, K.; Hirooka, Y.; Sunagawa, K. Miso (Japanese soybean paste) soup attenuates salt-induced sympathoexcitation and left ventricular dysfunction in mice with chronic pressure overload. *Fukuoka Igaku Zasshi.* **2014**, *105*, 48–56. [PubMed]
84. Tsai, T.Y.; Chen, L.Y.; Pan, T.M. Effect of probiotic-fermented, genetically modified soy milk on hypercholesterolemia in hamsters. *J. Microbiol. Immunol. Infect.* **2014**, *47*, 1–8. [CrossRef] [PubMed]
85. Eser, O.; Songur, A.; Yaman, M.; Cosar, M.; Fidan, H.; Sahin, O.; Mollaoglu, H.; Buyukbas, S. The protective effect of avocado soybean unsaponifilables on brain ischemia/reperfusion injury in rat prefrontal cortex. *Br. J. Neurosurg.* **2011**, *25*, 701–706. [CrossRef] [PubMed]
86. Zhang, M.; Cai, S.; Ma, J. Evaluation of cardio-protective effect of soybean oligosaccharides. *Gene* **2015**, *555*, 329–334. [CrossRef] [PubMed]
87. Jamarkattel-Pandit, N.; Pandit, N.R.; Kim, M.Y.; Park, S.H.; Kim, K.S.; Choi, H.; Kim, H.; Bu, Y. Neuroprotective effect of defatted sesame seeds extract against in vitro and in vivo ischemic neuronal damage. *Planta Med.* **2010**, *76*, 20–26. [CrossRef] [PubMed]
88. Lee, K.; Jo, I.Y.; Park, S.H.; Kim, K.S.; Bae, J.; Park, J.W.; Lee, B.J.; Choi, H.Y.; Bu, Y. Defatted sesame seed extract reduces brain oedema by regulating aquaporin 4 expression in acute phase of transient focal cerebral ischaemia in rat. *Phytother. Res.* **2012**, *26*, 1521–1527. [CrossRef] [PubMed]
89. Fujiwara, Y.; Kiyota, N.; Hori, M.; Matsushita, S.; Iijima, Y.; Aoki, K.; Shibata, D.; Takeya, M.; Ikeda, T.; Nohara, T.; et al. Esculeogenin a, a new tomato sapogenol, ameliorates hyperlipidemia and atherosclerosis in ApoE-deficient mice by inhibiting ACAT. *Arterioscl. Throm. Vas.* **2007**, *27*, 2400–2406. [CrossRef] [PubMed]
90. Fujiwara, Y.; Kiyota, N.; Tsurushima, K.; Yoshitomi, M.; Horlad, H.; Ikeda, T.; Nohara, T.; Takeya, M.; Nagai, R. Tomatidine, a tomato sapogenol, ameliorates hyperlipidemia and atherosclerosis in ApoE-Deficient mice by inhibiting Acyl-CoA:cholesterol acyl-transferase (ACAT). *J. Agric. Food Chem.* **2012**, *60*, 2472–2479. [CrossRef] [PubMed]
91. Parvin, R.; Akhter, N. Protective effect of tomato against adrenaline-induced myocardial infarction in rats. *Bangladesh Med. Res. Counc. Bull.* **2008**, *34*, 104–108. [CrossRef] [PubMed]
92. Yoshimura, M.; Toyoshi, T.; Sano, A.; Izumi, T.; Fujii, T.; Konishi, C.; Inai, S.; Matsukura, C.; Fukuda, N.; Ezura, H.; et al. Antihypertensive effect of a gamma-aminobutyric acid rich tomato cultivar 'DG03–9' in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2010**, *58*, 615–619. [CrossRef] [PubMed]

93. Vilahur, G.; Cubedo, J.; Padro, T.; Casani, L.; Mendieta, G.; Gonzalez, A.; Badimon, L. Intake of cooked tomato sauce preserves coronary endothelial function and improves apolipoprotein A-I and apolipoprotein J protein profile in high-density lipoproteins. *Transl. Res.* **2015**, *166*, 44–56. [CrossRef] [PubMed]
94. Tang, Y.N.; He, X.C.; Ye, M.; Huang, H.; Chen, H.L.; Peng, W.L.; Zhao, Z.Z.; Yi, T.; Chen, H.B. Cardioprotective effect of total saponins from three medicinal species of Dioscorea against isoprenaline-induced myocardial ischemia. *J. Ethnopharmacol.* **2015**, *175*, 451–455. [CrossRef] [PubMed]
95. Zhang, X.X.; Chen, L.; Liu, J.L.; Ito, Y.; He, J.; Sun, W.J. Neuroprotection of total steroid saponins from *Dioscorea zingiberensis* against transient focal cerebral ischemia-reperfusion injury in rats via anti-inflammatory and antiapoptotic effects. *Planta Med.* **2014**, *80*, 1597–1604. [CrossRef] [PubMed]
96. Gong, G.; Qin, Y.; Huang, W. Anti-thrombosis effect of diosgenin extract from *Dioscorea zingiberensis* C.H. Wright in vitro and in vivo. *Phytomedicine* **2011**, *18*, 458–463. [CrossRef] [PubMed]
97. Chen, C.T.; Wang, Z.H.; Hsu, C.C.; Lin, H.H.; Chen, J.H. In vivo protective effects of diosgenin against doxorubicin-induced cardiotoxicity. *Nutrients* **2015**, *7*, 4938–4954. [CrossRef] [PubMed]
98. Jayachandran, K.S.; Vasanthi, H.R.; Rajamanickama, G.V. Flavonoid rich fraction of *Dioscorea bulbifera* Linn. (Yam) enhances mitochondrial enzymes and antioxidant status and thereby protects heart from isoproterenol induced myocardial infarction. *Curr. Pharm. Biotechnol.* **2010**, *11*, 887–894. [CrossRef] [PubMed]
99. Koo, H.J.; Park, H.J.; Byeon, H.E.; Kwak, J.H.; Um, S.H.; Kwon, S.T.; Rhee, D.K.; Pyo, S. Chinese yam extracts containing beta-Sitosterol and ethyl linoleate protect against atherosclerosis in apolipoprotein E-Deficient mice and inhibit muscular expression of VCAM-1 in vitro. *J. Food Sci.* **2014**, *79*, H719–H729. [CrossRef] [PubMed]
100. Chang, W.C.; Yu, Y.M.; Wu, C.H.; Tseng, Y.H.; Wu, K.Y. Reduction of oxidative stress and atherosclerosis in hyperlipidemic rabbits by *Dioscorea rhizome*. *Can. J. Physiol. Pharmacol.* **2005**, *83*, 423–430. [CrossRef] [PubMed]
101. Amat, N.; Amat, R.; Abdureyim, S.; Hoxur, P.; Osman, Z.; Mamut, D.; Kijjoa, A. Aqueous extract of dioscorea opposita thunb. Normalizes the hypertension in 2K1C hypertensive rats. *BMC Complement. Altern. Med.* **2014**, *14*. [CrossRef] [PubMed]
102. Jaiswal, N.; Rizvi, S.I. Onion extract (*Allium cepa* L.), quercetin and catechin up-regulate paraoxonase 1 activity with concomitant protection against low-density lipoprotein oxidation in male Wistar rats subjected to oxidative stress. *J. Sci. Food Agric.* **2014**, *94*, 2752–2757. [CrossRef] [PubMed]
103. Li, W.; Tang, C.; Jin, H.; Du, J. Effects of onion extract on endogenous vascular H2S and adrenomedulin in rat atherosclerosis. *Curr. Pharm. Biotechnol.* **2011**, *12*, 1427–1439. [CrossRef] [PubMed]
104. Briggs, W.H.; Folts, J.D.; Osman, H.E.; Goldman, I.L. Administration of raw onion inhibits platelet-mediated thrombosis in dogs. *J. Nutr.* **2001**, *131*, 2619–2622. [PubMed]
105. Yamada, K.; Naemura, A.; Sawashita, N.; Noguchi, Y.; Yamamoto, J. An onion variety has natural antithrombotic effect as assessed by thrombosis/thrombolysis models in rodents. *Thromb. Res.* **2004**, *114*, 213–220. [CrossRef] [PubMed]
106. Lee, S.M.; Moon, J.; Chung, J.H.; Cha, Y.J.; Shin, M.J. Effect of quercetin-rich onion peel extracts on arterial thrombosis in rats. *Food Chem. Toxicol.* **2013**, *57*, 99–105. [CrossRef] [PubMed]
107. Sakai, Y.; Murakami, T.; Yamamoto, Y. Antihypertensive effects of onion on NO synthase inhibitor-induced hypertensive rats and spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1305–1311. [CrossRef] [PubMed]
108. Naseri, M.K.; Arabian, M.; Badavi, M.; Ahangarpour, A. Vasorelaxant and hypotensive effects of *Allium cepa* peel hydroalcoholic extract in rat. *Pak. J. Biol. Sci.* **2008**, *11*, 1569–1575. [CrossRef] [PubMed]
109. Moghadam, M.H.; Imenshahidi, M.; Mohajeri, S.A. Antihypertensive effect of celery seed on rat blood pressure in chronic administration. *J. Med. Food* **2013**, *16*, 558–563. [CrossRef] [PubMed]
110. Si, Y.; Guo, S.; Fang, Y.; Qin, S.; Li, F.; Zhang, Y.; Jiao, P.; Zhang, C.; Gao, L. Celery seed extract blocks peroxide injury in macrophages via Notch1/NF-kappaB pathway. *Am. J. Chin. Med.* **2015**, *43*, 443–455. [CrossRef] [PubMed]
111. Dianat, M.; Veisi, A.; Ahangarpour, A.; Moghaddam, H.F. The effect of hydro-alcoholic celery (Apiumgraveolens) leaf extract on cardiovascular parameters and lipid profile in animal model of hypertension induced by fructose. *Avicenna J. Phytomed.* **2015**, *5*, 203–209. [PubMed]

112. Sanae, M.; Yasuo, A. Green asparagus (*Asparagus officinalis*) prevented hypertension by an inhibitory effect on angiotensin-converting enzyme activity in the kidney of spontaneously hypertensive rats. *J. Agric. Food Chem.* **2013**, *61*, 5520–5525. [CrossRef] [PubMed]
113. Nicolle, C.; Cardinault, N.; Gueux, E.; Jaffrelo, L.; Rock, E.; Mazur, A.; Amouroux, P.; Remesy, C. Health effect of vegetable-based diet: Lettuce consumption improves cholesterol metabolism and antioxidant status in the rat. *Clin. Nutr.* **2004**, *23*, 605–614. [CrossRef] [PubMed]
114. Johnson, M.; Pace, R.D.; Dawkins, N.L.; Willian, K.R. Diets containing traditional and novel green leafy vegetables improve liver fatty acid profiles of spontaneously hypertensive rats. *Lipids Health Dis.* **2013**, *12*. [CrossRef] [PubMed]
115. Emran, T.B.; Rahman, M.A.; Uddin, M.M.; Rahman, M.M.; Uddin, M.Z.; Dash, R.; Layzu, C. Effects of organic extracts and their different fractions of five Bangladeshi plants on in vitro thrombolysis. *BMC Complement. Altern. Med.* **2015**, *15*. [CrossRef] [PubMed]
116. Yamada, Y.; Iwasaki, M.; Usui, H.; Ohinata, K.; Marczak, E.D.; Lipkowski, A.W.; Yoshikawa, M. Rapakinin, an anti-hypertensive peptide derived from rapeseed protein, dilates mesenteric artery of spontaneously hypertensive rats via the prostaglandin IP receptor followed by CCK(1) receptor. *Peptides* **2010**, *31*, 909–914. [CrossRef] [PubMed]
117. He, R.; Malomo, S.A.; Girgih, A.T.; Ju, X.; Aluko, R.E. Glycinyl-histidinyl-serine (GHS), a novel rapeseed protein-derived peptide has blood pressure-lowering effect in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2013**, *61*, 8396–8402. [CrossRef] [PubMed]
118. He, R.; Alashi, A.; Malomo, S.A.; Girgih, A.T.; Chao, D.; Ju, X.; Aluko, R.E. Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chem.* **2013**, *141*, 153–159. [CrossRef] [PubMed]
119. Breitbart, E.; Lomnitski, L.; Nyska, A.; Malik, Z.; Bergman, M.; Sofer, Y.; Haseman, J.K.; Grossman, S. Effects of water-soluble antioxidant from spinach, NAO, on doxorubicin-induced heart injury. *Hum. Exp. Toxicol.* **2001**, *20*, 337–345. [CrossRef] [PubMed]
120. Yang, Y.; Marczak, E.D.; Yokoo, M.; Usui, H.; Yoshikawa, M. Isolation and antihypertensive effect of angiotensin I-converting enzyme (ACE) inhibitory peptides from spinach Rubisco. *J. Agric. Food Chem.* **2003**, *51*, 4897–4902. [CrossRef] [PubMed]
121. Yang, Y.; Marczak, E.D.; Usui, H.; Kawamura, Y.; Yoshikawa, M. Antihypertensive properties of spinach leaf protein digests. *J. Agric. Food Chem.* **2004**, *52*, 2223–2225. [CrossRef] [PubMed]
122. Kwon, Y.I.; Apostolidis, E.; Kim, Y.C.; Shetty, K. Health benefits of traditional corn, beans, and pumpkin: In vitro studies for hyperglycemia and hypertension management. *J. Med. Food* **2007**, *10*, 266–275. [CrossRef] [PubMed]
123. Gilani, A.H.; Shaheen, F.; Saeed, S.A.; Bibi, S.; Irfanullah; Sadiq, M.; Faizi, S. Hypotensive action of coumarin glycosides from *Daucus carota*. *Phytomedicine* **2000**, *7*, 423–426. [CrossRef]
124. Nicolle, C.; Gueux, E.; Lab, C.; Jaffrelo, L.; Rock, E.; Mazur, A.; Amouroux, P.; Remesy, C. Lyophilized carrot ingestion lowers lipemia and beneficially affects cholesterol metabolism in cholesterol-fed C57BL/6J mice. *Eur. J. Nutr.* **2004**, *43*, 237–245. [CrossRef] [PubMed]
125. Yamamoto, J.; Naemura, A.; Ijiri, Y.; Ogawa, K.; Suzuki, T.; Shimada, Y.; Giddings, J.C. The antithrombotic effects of carrot filtrates in rats and mice. *Blood Coagul. Fibrinolysis* **2008**, *19*, 785–792. [CrossRef] [PubMed]
126. Wu, L.; Noyan, A.M.; Facci, M.; Wang, R.; Paterson, P.G.; Ferrie, A.; Juurlink, B.H. Dietary approach to attenuate oxidative stress, hypertension, and inflammation in the cardiovascular system. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7094–7099. [CrossRef] [PubMed]
127. Mukherjee, S.; Gangopadhyay, H.; Das, D.K. Broccoli: A unique vegetable that protects mammalian hearts through the redox cycling of the thioredoxin superfamily. *J. Agric. Food Chem.* **2008**, *56*, 609–617. [CrossRef] [PubMed]
128. Akhlaghi, M.; Bandy, B. Dietary broccoli sprouts protect against myocardial oxidative damage and cell death during ischemia-reperfusion. *Plant Foods Hum. Nutr.* **2010**, *65*, 193–199. [CrossRef] [PubMed]
129. Mukherjee, S.; Lekli, I.; Ray, D.; Gangopadhyay, H.; Raychaudhuri, U.; Das, D.K. Comparison of the protective effects of steamed and cooked broccolis on ischaemia-reperfusion-induced cardiac injury. *Br. J. Nutr.* **2010**, *103*, 815–823. [CrossRef] [PubMed]
130. Yang, Y.; Tao, G.; Liu, P.; Liu, J. Peptide with angiotensin I-converting enzyme inhibitory activity from hydrolyzed corn gluten meal. *J. Agric. Food Chem.* **2007**, *55*, 7891–7895. [CrossRef] [PubMed]

131. Shindo, M.; Kasai, T.; Abe, A.; Kondo, Y. Effects of dietary administration of plant-derived anthocyanin-rich colors to spontaneously hypertensive rats. *J. Nutr. Sci. Vitaminol.* **2007**, *53*, 90–93. [CrossRef] [PubMed]
132. Toufektsian, M.C.; de Lorgeril, M.; Nagy, N.; Salen, P.; Donati, M.B.; Giordano, L.; Mock, H.P.; Peterek, S.; Matros, A.; Petroni, K.; et al. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. *J. Nutr.* **2008**, *138*, 747–752. [PubMed]
133. Masini, E.; Pierpaoli, S.; Marzocca, C.; Mannaioni, P.F.; Pietrangeli, P.; Mateescu, M.A.; Zelli, M.; Federico, R.; Mondovi, B. Protective effects of a plant histaminase in myocardial ischaemia and reperfusion injury in vivo. *Biochem. Biophys. Res. Commun.* **2003**, *309*, 432–439. [CrossRef] [PubMed]
134. Masini, E.; Cuzzocrea, S.; Bani, D.; Mazzon, E.; Muja, C.; Mastroianni, R.; Fabrizi, F.; Pietrangeli, P.; Marcocci, L.; Mondovi, B.; et al. Beneficial effects of a plant histaminase in a rat model of splanchnic artery occlusion and reperfusion. *Shock* **2007**, *27*, 409–415. [CrossRef] [PubMed]
135. Burguieres, E.; Mccue, P.; Kwon, Y.I.; Shetty, K. Health-related functionality of phenolic-enriched pea sprouts in relation to diabetes and hypertension management. *J. Food Biochem.* **2008**, *32*, 3–14. [CrossRef]
136. Aukema, H.M.; Gauthier, J.; Roy, M.; Jia, Y.; Li, H.; Aluko, R.E. Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. *Mol. Nutr. Food Res.* **2011**, *55*, 1044–1051. [CrossRef] [PubMed]
137. Parolini, C.; Manzini, S.; Busnelli, M.; Rigamonti, E.; Marchesi, M.; Diani, E.; Sirtori, C.R.; Chiesa, G. Effect of the combinations between pea proteins and soluble fibres on cholesterolaemia and cholesterol metabolism in rats. *Br. J. Nutr.* **2013**, *110*, 1394–1401. [CrossRef] [PubMed]
138. Ma, L.; Grann, K.; Li, M.; Jiang, Z. A pilot study to evaluate the effect of soy isolate protein on the serum lipid profile and other potential cardiovascular risk markers in moderately hypercholesterolemic Chinese adults. *Ecol. Food Nutr.* **2011**, *50*, 473–485. [CrossRef] [PubMed]
139. Rebholz, C.M.; Reynolds, K.; Wofford, M.R.; Chen, J.; Kelly, T.N.; Mei, H.; Whelton, P.K.; He, J. Effect of soybean protein on novel cardiovascular disease risk factors: A randomized controlled trial. *Eur. J. Clin. Nutr.* **2013**, *67*, 58–63. [CrossRef] [PubMed]
140. Dettmer, M.; Alekel, D.L.; Lasrado, J.A.; Messina, M.; Carriquiry, A.; Heiberger, K.; Stewart, J.W.; Franke, W. The effect of soy protein beverages on serum cell adhesion molecule concentrations in prehypertensive/stage 1 hypertensive individuals. *J. Am. Coll. Nutr.* **2012**, *31*, 100–110. [CrossRef] [PubMed]
141. Liu, Z.M.; Ho, S.C.; Chen, Y.M.; Woo, J. A six-month randomized controlled trial of whole soy and isoflavones daidzein on body composition in equol-producing postmenopausal women with prehypertension. *J. Obes.* **2013**, *2013*. [CrossRef] [PubMed]
142. Liu, Z.M.; Ho, S.C.; Chen, Y.M.; Tomlinson, B.; Ho, S.; To, K.; Woo, J. Effect of whole soy and purified daidzein on ambulatory blood pressure and endothelial function–a 6-month double-blind, randomized controlled trial among Chinese postmenopausal women with prehypertension. *Eur. J. Clin. Nutr.* **2015**, *69*, 1161–1168. [CrossRef] [PubMed]
143. Hodis, H.N.; Mack, W.J.; Kono, N.; Azen, S.P.; Shoupe, D.; Hwang-Levine, J.; Petitti, D.; Whitfield-Maxwell, L.; Yan, M.; Franke, A.A.; et al. Isoflavone soy protein supplementation and atherosclerosis progression in healthy postmenopausal women: A randomized controlled trial. *Stroke* **2011**, *42*, 3168–3175. [CrossRef] [PubMed]
144. Liu, Z.M.; Ho, S.C.; Chen, Y.M.; Ho, Y.P. The effects of isoflavones combined with soy protein on lipid profiles, C-reactive protein and cardiovascular risk among postmenopausal Chinese women. *Nutr. Metab. Cardiovasc. Dis.* **2012**, *22*, 712–719. [CrossRef] [PubMed]
145. Wu, J.; Hodgson, J.M.; Puddey, I.B.; Belski, R.; Burke, V.; Croft, K.D. Sesame supplementation does not improve cardiovascular disease risk markers in overweight men and women. *Nutr. Metab. Cardiovasc.* **2009**, *19*, 774–780. [CrossRef] [PubMed]
146. Engelhard, Y.N.; Gazer, B.; Paran, E. Natural antioxidants from tomato extract reduce blood pressure inpatients with grade-1 hypertension: A double-blind, placebo-controlled pilot study. *Am. Heart J.* **2006**, *151*. [CrossRef] [PubMed]
147. Garcia-Alonso, F.J.; Jorge-Vidal, V.; Ros, G.; Periago, M.J. Effect of consumption of tomato juice enriched with *n*-3 polyunsaturated fatty acids on the lipid profile, antioxidant biomarker status, and cardiovascular disease risk in healthy women. *Eur. J. Nutr.* **2012**, *51*, 415–424. [CrossRef] [PubMed]

148. Valderas-Martinez, P.; Chiva-Blanch, G.; Casas, R.; Arranz, S.; Martinez-Huelamo, M.; Urpi-Sarda, M.; Torrado, X.; Corella, D.; Lamuela-Raventos, R.M.; Estruch, R. Tomato sauce enriched with olive oil exerts greater effects on cardiovascular disease risk factors than raw tomato and tomato sauce: A randomized trial. *Nutrients* **2016**, *8*, 170. [CrossRef] [PubMed]
149. Thies, F.; Masson, L.F.; Rudd, A.; Vaughan, N.; Tsang, C.; Brittenden, J.; Simpson, W.G.; Duthie, S.; Horgan, G.W.; Duthie, G. Effect of a tomato-rich diet on markers of cardiovascular disease risk in moderately overweight, disease-free, middle-aged adults: A randomized controlled trial. *Am. J. Clin. Nutr.* **2012**, *95*, 1013–1022. [CrossRef] [PubMed]
150. Christiansen, B.; Muguerza, N.B.; Petersen, A.M.; Kveiborg, B.; Madsen, C.R.; Thomas, H.; Ihlemann, N.; Sorensen, J.C.; Kober, L.; Sorensen, H.; et al. Ingestion of broccoli sprouts does not improve endothelial function in humans with hypertension. *PLoS ONE* **2010**, *5*. [CrossRef] [PubMed]
151. Medina, S.; Dominguez-Perles, R.; Moreno, D.A.; Garcia-Viguera, C.; Ferreres, F.; Gil, J.I.; Gil-Izquierdo, A. The intake of broccoli sprouts modulates the inflammatory and vascular prostanoids but not the oxidative stress-related isoprostanes in healthy humans. *Food Chem.* **2015**, *173*, 1187–1194. [CrossRef] [PubMed]
152. Nakayama, H.; Tsuge, N.; Sawada, H.; Higashi, Y. Chronic intake of onion extract containing quercetin improved postprandial endothelial dysfunction in healthy men. *J. Am. Coll. Nutr.* **2013**, *32*, 160–164. [CrossRef] [PubMed]
153. Bruell, V.; Burak, C.; Stoffel-Wagner, B.; Wolffram, S.; Nickenig, G.; Mueller, C.; Langguth, P.; Alteheld, B.; Fimmers, R.; Naaf, S.; et al. Effects of a quercetin-rich onion skin extract on 24 h ambulatory blood pressure and endothelial function in overweight-to-obese patients with (pre-) hypertension: A randomised double-blinded placebo-controlled cross-over trial. *Br. J. Nutr.* **2015**, *114*, 1263–1277. [CrossRef] [PubMed]
154. Rodriguez-Monforte, M.; Flores-Mateo, G.; Sanchez, E. Dietary patterns and CVD: A systematic review and meta-analysis of observational studies. *Br. J. Nutr.* **2015**, *114*, 1341–1359. [CrossRef] [PubMed]
155. Li, F.; Hou, L.N.; Chen, W.; Chen, P.L.; Lei, C.Y.; Wei, Q.; Tan, W.L.; Zheng, S.B. Associations of dietary patterns with the risk of all-cause, CVD and stroke mortality: A meta-analysis of prospective cohort studies. *Br. J. Nutr.* **2015**, *113*, 16–24. [CrossRef] [PubMed]
156. Schwingshackl, L.; Hoffmann, G. Diet quality as assessed by the healthy eating index, the alternate healthy eating index, the dietary approaches to stop hypertension score, and health outcomes: A systematic review and meta-analysis of cohort studies. *J. Acad. Nutr. Diet.* **2015**, *115*, 780–800. [CrossRef] [PubMed]
157. Siervo, M.; Lara, J.; Chowdhury, S.; Ashor, A.; Oggioni, C.; Mathers, J.C. Effects of the dietary approach to stop hypertension (DASH) diet on cardiovascular risk factors: A systematic review and meta-analysis. *Br. J. Nutr.* **2015**, *113*, 1–15. [CrossRef] [PubMed]
158. Grosso, G.; Marventano, S.; Yang, J.; Micek, A.; Pajak, A.; Scalfi, L.; Galvano, F.; Kales, S.N. A comprehensive meta-analysis on evidence of Mediterranean diet and cardiovascular disease: Are individual components equal? *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3218–3232. [CrossRef] [PubMed]
159. Sofi, F.; Macchi, C.; Abbate, R.; Gensini, G.F.; Casini, A. Mediterranean diet and health status: An updated meta-analysis and a proposal for a literature-based adherence score. *Public Health Nutr.* **2014**, *17*, 2769–2782. [CrossRef] [PubMed]
160. Rees, K.; Hartley, L.; Flowers, N.; Clarke, A.; Hooper, L.; Thorogood, M.; Stranges, S. Mediterranean dietary pattern for the primary prevention of cardiovascular disease. *Cochrane Database Syst. Rev.* **2013**, *8*. [CrossRef]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Long-Term Coffee Consumption Is Associated with Decreased Incidence of New-Onset Hypertension: A Dose–Response Meta-Analysis

Giuseppe Grosso [1,2,*], Agnieszka Micek [3], Justyna Godos [2], Andrzej Pajak [3], Salvatore Sciacca [2], Maira Bes-Rastrollo [4,5,6], Fabio Galvano [7] and Miguel A. Martinez-Gonzalez [4,5,6]

1. NNEdPro Global Centre for Nutrition and Health, St John's Innovation Centre, Cambridge CB4 0WS, UK
2. Integrated Cancer Registry of Catania-Messina-Siracusa-Enna, Azienda Ospedaliero-Universitaria Policlinico-Vittorio Emanuele, 95123 Catania, Italy; justyna.godos@student.uj.edu.pl (J.G.); sciacca@unict.it (S.S.)
3. Department of Epidemiology and Population Studies, Jagiellonian University Medical College, 31-008 Krakow, Poland; agnieszka.micek@uj.edu.pl (A.M.); andrzej.pajak@uj.ed.pl (A.P.)
4. Department of Preventive Medicine & Public Health, School of Medicine, University of Navarra, 31008 Pamplona, Spain; mbes@unav.es (M.B.-R.); mamartinez@unav.es (M.A.M.-G.)
5. IDISNA (Navarra's Health Research Institute, 31008 Pamplona, Spain
6. CIBERobn, Instituto de Salud Carlos III, 28029 Madrid, Spain
7. Department of Biomedical and Biotechnological Sciences, University of Catania, 95124 Catania, Italy; fgalvano@unict.it

* Correspondence: giuseppe.grosso@studium.unict.it; Tel.: +39-095-378-2182

Received: 29 May 2017; Accepted: 10 August 2017; Published: 17 August 2017

Abstract: Objective: To perform a dose–response meta-analysis of prospective cohort studies investigating the association between long-term coffee intake and risk of hypertension. Methods: An online systematic search of studies published up to November 2016 was performed. Linear and non-linear dose–response meta-analyses were conducted; potential evidence of heterogeneity, publication bias, and confounding effect of selected variables were investigated through sensitivity and meta-regression analyses. Results: Seven cohorts including 205,349 individuals and 44,120 cases of hypertension were included. In the non-linear analysis, there was a 9% significant decreased risk of hypertension per seven cups of coffee a day, while, in the linear dose–response association, there was a 1% decreased risk of hypertension for each additional cup of coffee per day. Among subgroups, there were significant inverse associations for females, caffeinated coffee, and studies conducted in the US with longer follow-up. Analysis of potential confounders revealed that smoking-related variables weakened the strength of association between coffee consumption and risk of hypertension. Conclusions: Increased coffee consumption is associated with a modest decrease in risk of hypertension in prospective cohort studies. Smoking status is a potential effect modifier on the association between coffee consumption and risk of hypertension.

Keywords: coffee; hypertension; risk; cohort; smoking; meta-analysis

1. Introduction

Coffee has been the focus of major attention due to its global consumption and impact on health [1]. Historically, coffee consumption was considered to have detrimental effects on health, particularly its contribution to high blood pressure [2]. However, coffee contains many other bioactive compounds, such as polyphenols, furans, pyrroles, and maltol, all of which have recently been hypothesized to have potential beneficial effects on human health [3,4]. Meta-analyses of prospective cohort studies show a J-shaped dose–response relationship between coffee consumption and decreased risk of cardiovascular disease (CVD), including coronary heart disease and stroke [5], and mortality [6]

suggesting that moderate consumption is key for its beneficial effects. Studies exploring acute events most likely triggered by coffee-dependent increase in blood pressure showed no substantial increase in mortality due to myocardial infarction [7] and atrial fibrillation [8]. Apart from cardiovascular health, coffee consumption has been hypothesized to affect a number of other conditions, including metabolic status [6] and risk of late-life cognitive impairment [9,10].

Overall, the benefits of coffee on human health seem evident; however, the association between coffee consumption and in particular risk of hypertension remains open for debate. Results from meta-analyses of randomized controlled trials testing the effects of caffeine intake and decaffeinated coffee on blood pressure were null and showed no increase in blood pressure [11,12]; however, these studies simply evaluated the effect of the caffeine in coffee and not of coffee itself. Due to the relatively short-term follow-up, previous studies mostly emphasized the acute effects of coffee intake rather than its long-term effects on blood pressure. Two previous meta-analyses of prospective cohort studies [13,14] reported non-significant increased risk of hypertension associated with higher intake of coffee. However, those meta-analyses only included a few studies reporting dose–response analysis and did not correct for the number of individuals/cases/person-years, and potential confounding factors/effect modifiers, ultimately providing limited-quality evidence. In light of these limitations and the availability of newly published cohort studies, this study aims to conduct a dose–response meta-analysis of prospective cohort studies investigating coffee consumption and the risk of hypertension.

2. Materials and Methods

The design, analysis, and reporting of this study is compliant with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Table S1).

2.1. Study Selection

A systematic search of PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and EMBASE (http://www.embase.com/) databases was conducted for studies up to November 2016 using the following search strategy: ("coffee" (MeSH Terms) OR "coffee" (All Fields)) AND ("hypertension" (MeSH Terms) OR "hypertension" (All Fields)) AND (cohort (All Fields) OR ("longitudinal studies" (MeSH Terms) OR ("longitudinal" (All Fields) AND "studies" (All Fields)) OR "longitudinal studies" (All Fields) OR "prospective" (All Fields) OR cases (All Fields). The inclusion criteria consisted of studies that: (i) are prospective; (ii) evaluated the association of coffee intake and the risk of hypertension in individuals without hypertension at baseline; (iii) assessed and reported hazard ratios (HRs), risk ratios (RRs), or odds ratios (ORs) and 95% CI for hypertension; and (iv) assessed and reported a defined amount of coffee consumption (i.e., cups per day) for each category of exposure. Reference lists of the included studies were also searched for any additional study not previously identified. For papers that studied the same cohort, only the study that included the entire cohort or had the longest follow-up was included.

2.2. Data Extraction

Data were extracted by two independent investigators (G.G. and A.M.) using a standardized extraction form. Discrepancies on the included studies were discussed and resolved by consensus. The following information was collected: (i) first author name; (ii) year of publication; (iii) study cohort name and country; (iv) total number, sex, and age (mean or range) of participants; (v) follow-up period; (vi) distributions of cases and person-years, HRs/RRs/ORs and 95% CIs for all categories of exposure; (vii) coffee intake range for each category of exposure; (viii) adjusted covariates; (ix) prevalence of smokers, diabetic subjects, and participants with low physical activity for each category of exposure; and (x) baseline mean/median systolic and diastolic blood pressure, age, body mass index (BMI), and daily intake of sodium and potassium for each category of coffee intake.

2.3. Study Quality Assessment

The quality of each study was assessed according to the Newcastle-Ottawa Quality Assessment Scale [15] composed of 3 variables: selection (4 points), comparability (2 points), and outcome (3 points) resulting in a total score of 9 points (9 representing the highest quality). Studies scoring 7–9 points, 3–6 points, and 0–3 points were identified as high, moderate, and low quality, respectively.

2.4. Statistical Analysis

In this meta-analysis, ORs and HRs referring to new onset incident cases of hypertension were deemed equivalent to risk ratios (RRs) [16]. When coffee consumption was illustrated by ranges of intake, the midpoint of the range was used. When the highest category was open-ended, we assumed that the width of the upper category was the same as the adjacent category. When the lowest category was open-ended, we set the lower boundary to zero. Two-stage random-effects dose–response meta-analysis was performed to examine linear and non-linear relationship between coffee intake and risk of developing hypertension during follow-up.

First, generalized least-squares (GLS) were used to calculate study-specific coefficients across categories of coffee intake accounting for the correlation within each set of RRs [17,18]. Non-linear dose–response analysis was modeled using restricted cubic splines with 3 knots at fixed percentiles (25%, 50%, and 75%) of the distribution [19]. Coefficients estimated within each study were combined by performing random-effects meta-analysis. DerSimonian and Laird's method was used for linear dose–response meta-analysis and, in non-linear dose–response meta-analysis, the multivariate extension of the method of moments was used to estimate the RRs. We tested whether the 2 regression coefficients were simultaneously equal to zero and calculated a p-value for non-linearity by testing whether the coefficient of the second spline was equal to zero. A sensitivity analysis by excluding one study at the time was conducted to determine the stability and robustness of the results.

To test for potential confounders, subgroup analyses by sex, type of coffee, and geographical area were performed. To further investigate whether other unmeasured potential confounders should be included in the interpretation of the results, we investigated the distribution of systolic blood pressure, diastolic blood pressure, age, BMI, daily intake of sodium and potassium, percentage of smokers, participants with low physical activity and those with type-2 diabetes across categories of coffee consumption. For this purpose, a separate two-stage bivariate meta-analysis was performed for each confounder variable to determine its association with coffee intake [20]. First, linear regression coefficients (slope and intercept) between coffee intake and the potential confounders above were estimated. Second, we used GLS to synthesize these intercepts and slope coefficients, accounting for the corresponding variance-covariance matrices [21]. To determine the significance of the proposed confounders on the association of coffee consumption and risk of hypertension, a meta-regression analysis was conducted. Specifically, we used the joint slope coefficient of the association as the moderator in the meta-regression analysis. Percentage of smokers was also used as moderator.

Publication bias was assessed using Egger's regression test while the Cochran Q-test tested for statistical heterogeneity (statistical significance is defined as a p value less than 0.10) and quantified through the multivariate generalization of the I^2 statistic (no, low, medium, and high heterogeneity were defined by I^2 values <25%, 25–50%, 50–75%, and >75%, respectively). All analyses were performed on R, software version 3.0.3, dosresmeta and mvmeta packages (Development Core Team, Vienna, Austria).

3. Results

3.1. Study Characteristics

The selection process of studies included in the meta-analysis is illustrated in Figure 1. Out of the 139 studies screened, six studies [22–27] involving seven cohorts, 205,349 individuals, and 44,120 cases of hypertension were included (Table 1). The sample of three studies included general population [24–26], registered US nurses (2 cohorts) [23], post-menopausal women [27], and post-graduate students [22].

Four cohorts were based in the US [22,23,27] and three in Europe [24–26]. Sex-specific (both male and female) risk estimates were provided by three studies [24–26], while two papers [23,27] studied only females and one only males [22]. Follow-up periods ranged an average of 3–33 years. All studies adjusted for variables that affect the risk of hypertension, including age, gender, BMI, and smoking status. Overall, the quality of all included studies was high (data not shown).

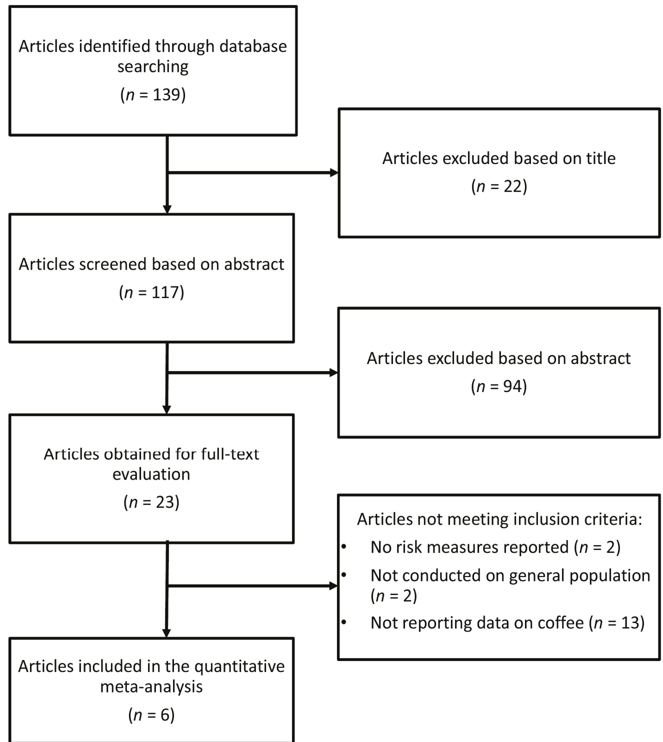


Figure 1. Flowchart of study selection for inclusion in the meta-analysis.

Table 1. Main characteristics of prospective cohort studies investigating the association of coffee consumption and risk of hypertension.

Author, Year	Name of Cohort (Years of Recruitment), Country	Cohort Size	No. of Cases	Gender	Exposure Variables	Follow-Up Duration	Adjusted Variables
Grosso, 2016 (21)	HAPIEE Cohort Study (2002–2008), Poland	2725	1735	MF	Caffeinated and decaffeinated coffee	5 y	Age, gender, education, occupation, BMI, alcohol consumption, smoking status, physical activity level, past history of CVD and diabetes at baseline, cholesterol therapy at baseline, total energy intake, vitamin supplement use, oral contraceptive use, and intake of sodium and potassium.
Rhee, 2016 (22)	WHI Observational Study, (1993), US	29,985	5566	F	Caffeinated coffee	3 y	Age, baseline blood pressure, BMI, physical activity, hormone replacement therapy, alcohol consumption, smoking status, total calorie intake, and intake of sodium, magnesium, calcium, potassium, and phosphorus as time-varying covariates.
Uiterwaal, 2007 (20)	DCS (1987–2002), The Netherlands	6368	956	MF	Caffeinated and decaffeinated coffee	11 y	Baseline age, sex, height and weight, smoking, alcohol intake, tea intake, education level, occupational status, and total energy intake.
Hu, 2007 (19)	Four independent surveys, (1982–2002), Finland	24,710	2505	MF	Caffeinated and decaffeinated coffee	13.2 y	Year, education, leisure-time physical activity, smoking status, alcohol consumption, tea consumption, frequency of vegetable, fruit, sausage, and bread consumption, BMI, history of diabetes and total cholesterol, and baseline systolic blood pressure.
Winkelmayer, 2005 (18)	NHS I (1976–2002), US	53,175	19,541	F	Caffeinated coffee	12 y	Age, BMI, alcohol consumption, family history of hypertension, physical activity, and smoking status, and intake of other beverages.
Winkelmayer, 2005 (18)	NHS II (1989–2003), US	87,369	13,536	F	Caffeinated coffee	12 y	Age, BMI, alcohol consumption, family history of hypertension, oral contraceptive use, physical activity, and smoking status, and intake of other beverages.
Klag, 2002 (17)	JHPS (1947–1995), US	1017	281	M	Caffeinated coffee	33 y	Parental history of hypertension, time-dependent number of cigarettes smoked, alcohol intake, physical activity, BMI.

DCS: Doetinchem Cohort Study; HAPIEE: Health, Alcohol and Psychosocial factors In Eastern Europe; JHPS: John Hopkins Precursors Study; NHS: Nurses' Health Study; WHI: Women's Health Initiative Observational Study; y: year.

3.2. Coffee Consumption and Risk of Hypertension

The dose–response regression in Figure 2A illustrates a non-significant decrease in risk of hypertension with up to six cups/day and a 9% significant decrease in risk observed for seven cups/day (RR = 0.91, 95% CI: 0.83–1.00), with moderate evidence of heterogeneity (I^2 = 51%, $P_{heterogeneity}$ = 0.004) and no publication bias (P_{Egger} = 0.755) (Table 2). After exclusion of one study [24], the association between coffee consumption and risk of hypertension became significant with no evidence of heterogeneity and publication bias (Table S2). When linear dose–response regression (test for non-linearity resulted not significant) was modeled, similar risk estimates resulted (Figure 2B), where there was a 1% decreased risk of hypertension with each additional cup of coffee per day (RR = 0.99, 95% CI: 0.98, 1.00; I^2 = 21%, $P_{heterogeneity}$ = 0.241; Figure 3). Stratified analyses by sex, geographical area, type of coffee consumed, and length of follow-up were limited by the small number of datasets; however, significant inverse associations were observed in studies conducted in the US, and those that studied only females, caffeinated coffee, and had longer follow-up (Table 2).

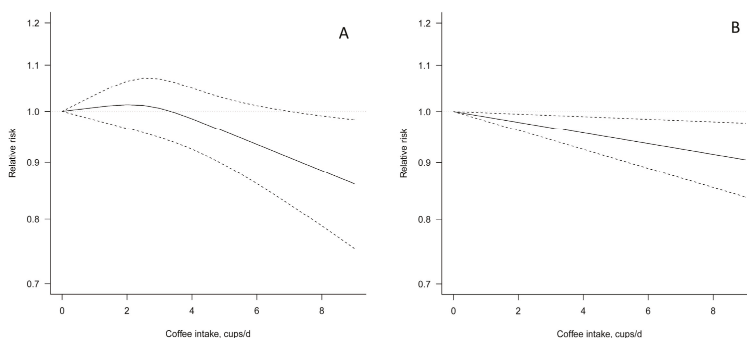


Figure 2. Dose–response association between coffee consumption and risk of hypertension: (**A**) non-linear association; and (**B**) linear association. Solid lines represent risk ratio, dashed lines represent 95% confidence intervals.

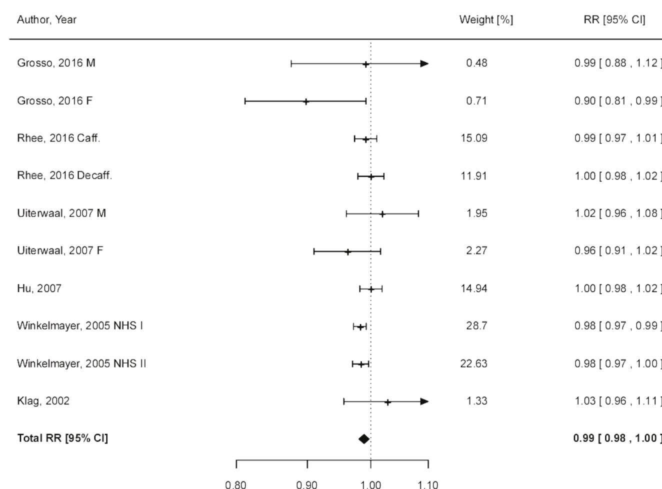


Figure 3. Forest plot illustrating risk of hypertension due to increase intake of one-cup of coffee per day in cohort studies.

Table 2. Dose–response meta-analysis of coffee consumption and risk of hypertension, stratified by selected variables.

	No. of Datasets (No. of Studies)	Coffee Intake (Cups/Day)								I^2	$P_{heterogeneity}$
		0	1	2	3	4	5	6	7		
All											
Non-linear	10 (6)	Ref.	1.01 (0.98–1.03)	1.01 (0.97–1.06)	1.01 (0.95–1.07)	0.98 (0.92–1.05)	0.96 (0.89–1.03)	0.93 (0.86–1.01)	0.91 (0.83–1.00)	51%	0.004
Linear	10 (6)	Ref.	0.99 (0.98–1.00)	0.98 (0.96–0.99)	0.97 (0.94–0.99)	0.96 (0.92–0.99)	0.95 (0.91–0.99)	0.94 (0.89–0.98)	0.92 (0.87–0.98)	21%	0.241
Males											
Non-linear	3 (3)	Ref.	1.09 (0.94–1.27)	1.19 (0.89–1.58)	1.24 (0.88–1.76)	1.23 (0.89–1.69)	1.19 (0.9–1.59)	1.16 (0.86–1.55)	1.12 (0.79–1.59)	0%	0.458
Linear	3 (3)	Ref.	1.02 (0.98–1.06)	1.04 (0.95–1.13)	1.06 (0.93–1.21)	1.08 (0.91–1.28)	1.1 (0.89–1.36)	1.12 (0.86–1.45)	1.14 (0.84–1.55)	0%	0.458
Females											
Non-linear	4 (4)	Ref.	1.00 (0.97–1.03)	1.00 (0.94–1.05)	0.98 (0.92–1.04)	0.95 (0.91–0.99)	0.92 (0.88–0.95)	0.88 (0.84–0.94)	0.85 (0.78–0.93)	43%	0.059
Linear	6 (4)	Ref.	0.99 (0.98–0.99)	0.97 (0.95–0.99)	0.96 (0.93–0.98)	0.94 (0.91–0.98)	0.93 (0.89–0.97)	0.92 (0.87–0.97)	0.90 (0.85–0.96)	26%	0.232
Caffeinated coffee											
Non-linear	4 (3)	Ref.	1.00 (0.97–1.02)	0.99 (0.95–1.04)	0.98 (0.93–1.03)	0.95 (0.92–0.99)	0.92 (0.89–0.96)	0.9 (0.84–0.96)	0.87 (0.79–0.96)	44%	0.095
Linear	4 (3)	Ref.	0.98 (0.98–0.99)	0.97 (0.95–0.98)	0.95 (0.93–0.98)	0.94 (0.91–0.97)	0.92 (0.89–0.96)	0.91 (0.87–0.95)	0.9 (0.85–0.94)	0%	0.529
Europe											
Non-linear	5 (3)	Ref.	1.02 (0.9–1.16)	1.04 (0.81–1.33)	1.06 (0.76–1.48)	1.08 (0.8–1.47)	1.12 (0.92–1.35)	1.15 (0.98–1.35)	1.19 (0.91–1.55)	54%	0.025
Linear	5 (3)	Ref.	0.99 (0.96–1.02)	0.97 (0.91–1.04)	0.96 (0.87–1.05)	0.95 (0.83–1.07)	0.93 (0.79–1.09)	0.92 (0.76–1.11)	0.91 (0.72–1.13)	38%	0.166
US											
Non-linear	3 (3)	Ref.	1 (0.98–1.02)	1 (0.96–1.04)	0.98 (0.94–1.03)	0.96 (0.92–0.99)	0.92 (0.89–0.96)	0.89 (0.84–0.94)	0.86 (0.79–0.93)	36	0.130
Linear	5 (3)	Ref.	0.99 (0.98–0.99)	0.97 (0.96–0.99)	0.96 (0.94–0.98)	0.95 (0.92–0.97)	0.93 (0.9–0.97)	0.92 (0.88–0.96)	0.91 (0.86–0.95)	1%	0.398
Follow up >10 years											
Non-linear	6 (4)	Ref.	1.02 (0.99–1.04)	1.03 (0.98–1.09)	1.02 (0.96–1.10)	0.99 (0.91–1.08)	0.95 (0.85–1.05)	0.91 (0.79–1.03)	0.87 (0.74–1.01)	57%	0.01
Linear	6 (4)	Ref.	0.99 (0.98–1.00)	0.97 (0.96–0.99)	0.96 (0.93–0.99)	0.95 (0.91–0.99)	0.94 (0.89–0.98)	0.93 (0.87–0.98)	0.91 (0.85–0.98)	21%	0.277
Follow up <10 years											
Non-linear	4 (2)	Ref.	0.97 (0.91–1.04)	0.95 (0.85–1.07)	0.94 (0.83–1.07)	0.94 (0.83–1.07)	0.95 (0.83–1.08)	0.95 (0.82–1.1)	0.95 (0.80–1.12)	43%	0.101
Linear	4 (2)	Ref.	0.99 (0.97–1.01)	0.98 (0.94–1.02)	0.97 (0.92–1.04)	0.97 (0.89–1.05)	0.96 (0.86–1.06)	0.95 (0.84–1.07)	0.94 (0.82–1.09)	31%	0.225

3.3. Potential Confounding Factors

The distribution of systolic and diastolic blood pressure and percentage of smokers across categories of coffee consumption was explored in four studies where a significant association was evident only with diastolic blood pressure (Figure 4). The prevalence of smokers in the no-coffee consumption category was 16.8%, which increased by about 6.4% for each additional cup of coffee consumed per day (Table 3).

Figure 5 illustrates two meta-regression models to test the confounding role of smoking status on the association of coffee consumption and risk of hypertension, both of which resulted in insignificant associations. With prevalence of smokers as the moderator (Figure 5A), studies with lower prevalence showed that coffee consumption was associated with decreased risk of hypertension, while in studies with higher prevalence, coffee consumption was associated with increased risk. With slope coefficients as the moderator (Figure 5B), studies with no change in prevalence of smokers across categories of coffee consumption (lower slope values) showed that coffee consumption was associated with decreased risk of hypertension, while, in studies where increased coffee consumption was linearly associated with higher prevalence of smokers (higher slope values), coffee consumption was associated with increased risk of hypertension.

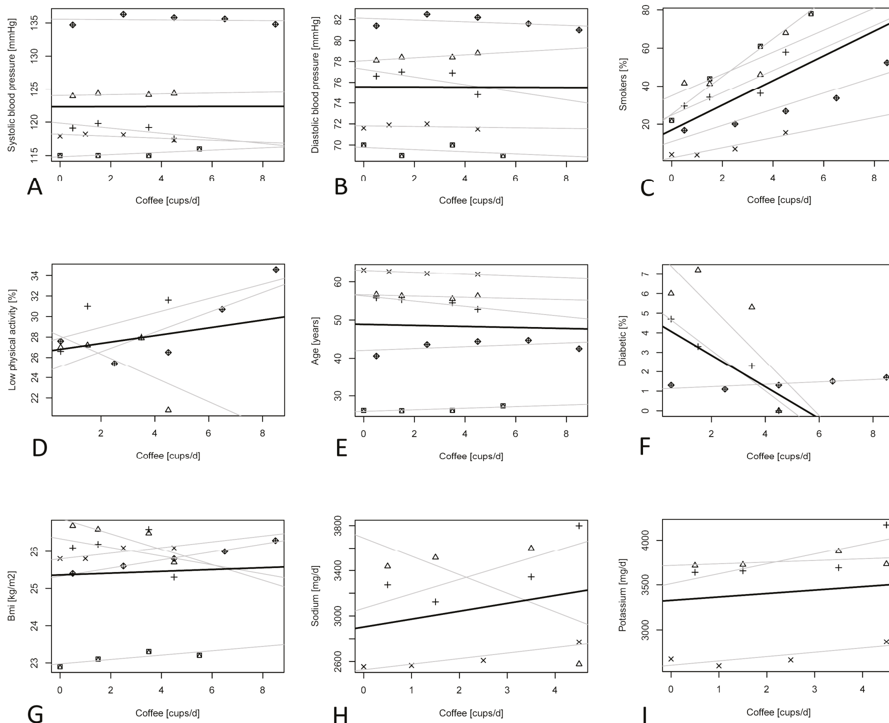


Figure 4. Scatter plots illustrating the relationship between coffee consumption (in cups per day) and baseline characteristics in the prospective cohorts: (**A**) systolic blood pressure; (**B**) diastolic blood pressure; (**C**) percentage of smokers; (**D**) percentage of low physically active individuals; (**E**) age; (**F**) percentage of diabetic individuals; (**G**) body mass index; (**H**) sodium intake; and (**I**) potassium intake. Symbols represent different cohorts; light lines represent linear regression coefficients of individual studies; bold lines represent summary estimates of average increase of each variable with increase in coffee intake. Cups/d: cups/day.

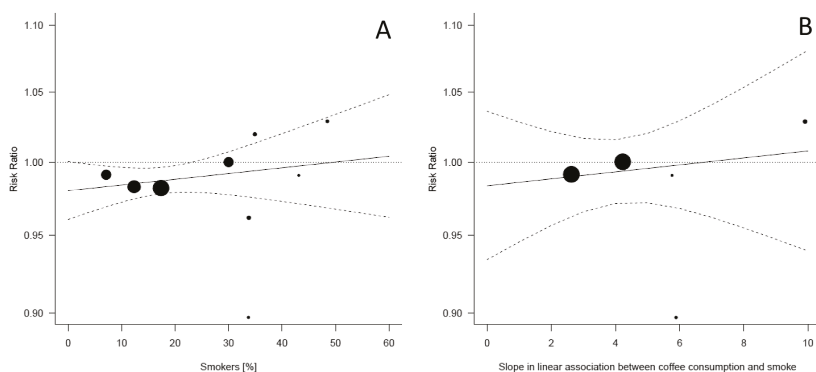


Figure 5. Risk of hypertension for linear increase of coffee consumption and selected smoking-related moderators: (**A**) total prevalence of smokers in the included cohorts; and (**B**) slope coefficients of the association between coffee intake and percentage of smokers in each category of exposure and random effects meta-regression. The area of each circle is inversely proportional to the variance of the log risk ratio estimate; continuous lines represent average risk ratios; and dashed lines represent corresponding 95% confidence intervals.

Table 3. Summary of the associations between coffee consumption and selected variables.

	No. of Datasets (No. of Studies)	Intercept (95% CI)	Slope per 1 Cup/Day (95% CI)	*p* for Slope
SBP (mmHg)	5 (4)	122.3 (115.2, 129.3)	0.01 (−0.12, 0.13)	0.9345
DBP (mmHg)	5 (4)	75.6 (71.2, 79.8)	−0.01 (−0.14, 0.12)	0.9131
Smokers (%)	5 (4)	16.9 (7.2, 26.7)	6.49 (3.77, 9.22)	<0.001
BMI	5 (4)	25.4 (24.1, 26.6)	0.02 (−0.08, 0.13)	0.6461
Age (year)	5 (4)	48.9 (35.9, 61.8)	−0.13 (−0.42, 0.15)	0.3565
Sodium (mg/day)	3 (2)	2903.2 (2480.9, 3325.6)	69.2 (37.0, 101.4)	<0.001
Potassium (mg/day)	3 (2)	3326.6 (2613.5, 4039.7)	38.0 (3.2, 72.8)	0.0326
Low PA (%)	3 (2)	26.6 (23.8, 29.4)	0.38 (−0.83, 1.59)	0.5391
Diabetic (%)	3 (2)	4.48 (0.51, 8.45)	−0.81 (−1.84, 0.22)	0.1218

BMI: body mass index; DBP: diastolic blood pressure; PA: physical activity; SBP: systolic blood pressure.

4. Discussion

This meta-analysis illustrated a linear association between increasing intake of coffee consumption and decreased risk of hypertension in six prospective cohort studies. However, when smoking status was included as a moderator in the meta-regression analyses, the association was weakened, suggesting that smoking may be an effect modifier. Compared to previous meta-analyses [13,14], this study included a larger sample (39,078 additional individuals and 8257 additional cases), dose–response analyses, and tested for potential confounders. The comparison of different categories of exposure (i.e., the variation of coffee intake across studies), may have biased the pooled risk estimates of previous meta-analyses of cohort studies. Differences in the results from this study compared to those from meta-analyses of randomized controlled trials [11,12] may be due to the potential long-term effect of coffee consumption captured only in cohort studies.

From the subgroup analyses, this study showed significant results in studies (or datasets) that only included women, were conducted in the US, and had a longer follow-up. Significant results seen in women and not in men could be attributed to increased prevalence of smoking in males accounting for 30% of all mortality in males 50–70 years [28]. To account for geographical differences in the results, the number of participants in US cohorts was three times more than the European cohorts. Thus, it can be inferred the number of individuals in the European cohorts was not large enough to obtain the expected effect size. This is in fact in line with the results obtained, as risk estimates in

both subgroups (US and European cohorts) were similar but the analysis of the European cohorts had larger confidence intervals. Furthermore, significant results seen in studies with longer follow-up seems intuitive since studies with longer follow-up more likely capture potential cases of chronic non-communicable disease.

Apart from those above, other reasons may account for the differences observed between subgroups (especially those between geographical areas), including variations in preferred coffee constituents, variations in brewing and in the actual amount of coffee contained in "a cup" across regions, and differences in population genetics. There is evidence that genetic factors related to target receptors and CYP1A2, such as the genetic polymorphism rs762551, also known as −136 C>A CYP1A2, encoding the CYP1A2*1F allele of the CYP1A2 gene, leads to slow metabolism of caffeine [29,30]. Carriers of the CYP1A2*1F allele have been demonstrated to be at higher risk of nonfatal myocardial infarction following intake of caffeinated coffee [31]. Similarly, the risk of sustained hypertension has been associated with CYP1A2*1F polymorphisms in a cohort of stage I hypertensive individuals [32]. Due to the variability of genetic polymorphisms of CYP1A2 enzyme, also related to ethnicity, it is plausible that there exist such genetic polymorphisms in the examined cohorts that result in the heterogeneity of our results, especially those found across geographical regions.

Another finding of this study was the potential effect modification of sodium intake on risk of hypertension associated with coffee consumption. Despite insufficient data for further examining this issue, three studies showed direct association between sodium intake and coffee consumption where increased intake of one cup of coffee per day was associated with 38 mg/day additional intake of sodium. It is known that increased sodium intake may be associated with increased fluid retention, but does not increase urine volume excretion, thus leading to a rise in body weight and blood pressure [33]. A comprehensive systematic review exploring data from 93 studies including more than 600 empirically-derived dietary patterns reported that coffee was part of a "healthy" dietary pattern in 4 studies and part of an "unhealthy" dietary pattern in 11 studies, suggesting that coffee drinkers may have, at least in part, unhealthier dietary choices, which may include higher sodium intake [34]. However, it is unclear whether the reported increase in sodium intake may have clinical relevance and more studies are needed to better explore this potential association.

The biological evidence supporting the association between coffee consumption and decreased risk of hypertension discounting caffeine intake has been investigated only during the last decade. The coffee content in phenolic compounds has been considered the main factor responsible for the beneficial effects on blood pressure mentioned previously [2]. The most studied compounds are chlorogenic acids, including metabolites ferulic acid, caffeic acid and quinic acid, which have all been reported to exert anti-hypertensive effects in experimental studies [35]. The main mechanisms of action rely on the anti-oxidant activity of chlorogenic acid, through its inhibition of the NAD(P)H oxidase expression and activity and by directly scavenging for free radicals [36]. Moreover, chlorogenic acid has been reported to stimulate nitric oxide production by the endothelial-dependent pathway, suggesting that vascular integrity, in particular, intact endothelium, is essential for chlorogenic acids to have a blood pressure lowering effect [37,38].

This meta-analysis has some limitations that should be addressed. The main issue in interpreting our findings depends on the potential confounders (smoking status, sodium intake) and genetic polymorphisms that are not explored in existing cohorts. We attempted an in-depth analysis on the role of smoking status, suggesting that it poses as an effect modifier. No data on genetic polymorphisms and only limited data on sodium intake were available and, hence, we were not able to analyze the potential effect modification of these variables, which may have weakened the observed results. Nevertheless, the large number of individuals included, the lack of evidence on heterogeneity, and the observed significant inverse association observed for the main effect variables all support the robustness of the findings. Another limitation in the papers included in this study is the lack of information on the type of coffee used (e.g., boiled, filtered, etc.) and on the actual amount of coffee used in one cup. The type of coffee consumed by US cohorts could be different from those consumed by European

cohorts, which contribute to the heterogeneous findings. Similarly, the amount of coffee added in a cup can vary across countries. One cup of coffee could refer to 150 mL or 300 mL which is another source of heterogeneity in these results. However, a previous meta-analysis that adjusted for the varying measurements of coffee showed identical results to the unadjusted measures on the outcome investigated [6].

Considering that the results from this meta-analysis may not account for the genetic variations contributing to the slow metabolism of caffeine, overall current evidence suggest that usual coffee consumption in the long-term is not a risk factor for developing hypertension in existing cohort studies. Rather results show an inverse association of increase coffee consumption and the risk of developing hypertension. Due to lack of long-term randomized controlled trials, results from cohort studies represent the most reliable available evidence when studying long-term effects of food on future health.

To date, our study provides a robust overview of the highest level of evidence that currently exists on the association of coffee consumption and risk of hypertension. Nevertheless, more cohort studies are needed to provide more in-depth and long-term evidence of this association. Additional factors such as smoking status, sodium intake and genetic polymorphisms need to be addressed in future studies to allow for stratified sub-group analyses. Further evidence on decaffeinated coffee could also provide information on the role of caffeine in such long-term outcomes.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/8/890/s1.

Acknowledgments: No funding. The authors are grateful to Shivani Bhat for the English language editing.

Author Contributions: The authors' responsibilities were as follows: G.G. conducted the literature search, extracted the data, prepared the first draft of the article, and contributed to the writing, editing, and proofreading of the final version of the article; A.M. conducted the statistical analyses and contributed to data interpretation; J.G. contributed to data acquisition and contributed to the writing, editing, and proofreading of the final version of the article; S.S., A.P. and M.B.-R. reviewed the manuscript; and F.G. and M.A.M.-G. provided insights for the interpretation of findings and equally contributed to the writing, editing, and proofreading of the final version of the article. All authors read and approved the final version of the article for publication.

Conflicts of Interest: All authors declare no conflict of interest.

References

1. Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, Caffeine, and Health Outcomes: An Umbrella Review. *Annu. Rev. Nutr.* **2017**, *37*. [CrossRef]
2. Godos, J.; Pluchinotta, F.R.; Marventano, S.; Buscemi, S.; Li Volti, G.; Galvano, F.; Grosso, G. Coffee components and cardiovascular risk: Beneficial and detrimental effects. *Int. J. Food Sci. Nutr.* **2014**, *65*, 925–936. [CrossRef] [PubMed]
3. Higdon, J.V.; Frei, B. Coffee and health: A review of recent human research. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 101–123. [CrossRef] [PubMed]
4. Buscemi, S.; Marventano, S.; Antoci, M.; Cagnetti, A.; Castorina, G.; Galvano, F.; Marranzano, M.; Mistretta, A. Coffee and metabolic impairment: An updated review of epidemiological studies. *NFS J.* **2016**, *3*, 1–7. [CrossRef]
5. Ding, M.; Bhupathiraju, S.N.; Satija, A.; van Dam, R.M.; Hu, F.B. Long-term coffee consumption and risk of cardiovascular disease: A systematic review and a dose-response meta-analysis of prospective cohort studies. *Circulation* **2014**, *129*, 643–659. [CrossRef] [PubMed]
6. Marventano, S.; Salomone, F.; Godos, J.; Pluchinotta, F.; Del Rio, D.; Mistretta, A.; Grosso, G. Coffee and tea consumption in relation with non-alcoholic fatty liver and metabolic syndrome: A systematic review and meta-analysis of observational studies. *Clin. Nutr.* **2016**, *35*, 1269–1281. [CrossRef] [PubMed]
7. Brown, O.I.; Allgar, V.; Wong, K.Y. Coffee reduces the risk of death after acute myocardial infarction: A meta-analysis. *Coron. Artery Dis.* **2016**, *27*, 566–572. [CrossRef] [PubMed]
8. Larsson, S.C.; Drca, N.; Jensen-Urstad, M.; Wolk, A. Coffee consumption is not associated with increased risk of atrial fibrillation: Results from two prospective cohorts and a meta-analysis. *BMC Med.* **2015**, *13*, 207. [CrossRef] [PubMed]

9. Panza, F.; Solfrizzi, V.; Barulli, M.R.; Bonfiglio, C.; Guerra, V.; Osella, A.; Seripa, D.; Sabba, C.; Pilotto, A.; Logroscino, G. Coffee, tea, and caffeine consumption and prevention of late-life cognitive decline and dementia: A systematic review. *J. Nutr. Health Aging* **2015**, *19*, 313–328. [CrossRef] [PubMed]
10. Solfrizzi, V.; Panza, F.; Imbimbo, B.P.; D'Introno, A.; Galluzzo, L.; Gandin, C.; Misciagna, G.; Guerra, V.; Osella, A.; Baldereschi, M.; et al. Coffee consumption habits and the risk of mild cognitive impairment: The italian longitudinal study on aging. *J. Alzheimers Dis.* **2015**, *47*, 889–899. [CrossRef] [PubMed]
11. Noordzij, M.; Uiterwaal, C.S.; Arends, L.R.; Kok, F.J.; Grobbee, D.E.; Geleijnse, J.M. Blood pressure response to chronic intake of coffee and caffeine: A meta-analysis of randomized controlled trials. *J. Hypertens.* **2005**, *23*, 921–928. [CrossRef] [PubMed]
12. Jee, S.H.; He, J.; Whelton, P.K.; Suh, I.; Klag, M.J. The effect of chronic coffee drinking on blood pressure: A meta-analysis of controlled clinical trials. *Hypertension* **1999**, *33*, 647–652. [CrossRef] [PubMed]
13. Steffen, M.; Kuhle, C.; Hensrud, D.; Erwin, P.J.; Murad, M.H. The effect of coffee consumption on blood pressure and the development of hypertension: A systematic review and meta-analysis. *J. Hypertens.* **2012**, *30*, 2245–2254. [CrossRef] [PubMed]
14. Zhang, Z.; Hu, G.; Caballero, B.; Appel, L.; Chen, L. Habitual coffee consumption and risk of hypertension: A systematic review and meta-analysis of prospective observational studies. *Am. J. Clin. Nutr.* **2011**, *93*, 1212–1219. [CrossRef] [PubMed]
15. Wells, G.; O'Connell, D.; Peterson, J.; Welch, V.; Losos, M.; Tugwell, P. *The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomised Studies in Meta-Analyses*; Ottawa Health Research Institute: Ottawa, ON, Canada, 1999.
16. Greenland, S. Quantitative methods in the review of epidemiologic literature. *Epidemiol. Rev.* **1987**, *9*, 1–30. [CrossRef]
17. Greenland, S.; Longnecker, M.P. Methods for trend estimation from summarized dose-response data, with applications to meta-analysis. *Am. J. Epidemiol.* **1992**, *135*, 1301–1309. [CrossRef] [PubMed]
18. Orsini, N.; Bellocco, R.; Greenland, S. Generalized least squares for trend estimation of summarized dose-response data. *Stata J.* **2006**, *6*, 40–57.
19. Orsini, N.; Li, R.; Wolk, A.; Khudyakov, P.; Spiegelman, D. Meta-analysis for linear and nonlinear dose-response relations: Examples, an evaluation of approximations, and software. *Am. J. Epidemiol.* **2012**, *175*, 66–73. [CrossRef] [PubMed]
20. Stukel, T.A.; Demidenko, E. Two-stage method of estimation for general linear growth curve models. *Biometrics* **1997**, *53*, 720–728. [CrossRef] [PubMed]
21. Becker, B.J.; Wu, M.-J. The synthesis of regression slopes in meta-analysis. *Stat. Sci.* **2007**, *22*, 414–429. [CrossRef]
22. Klag, M.J.; Wang, N.Y.; Meoni, L.A.; Brancati, F.L.; Cooper, L.A.; Liang, K.Y.; Young, J.H.; Ford, D.E. Coffee intake and risk of hypertension: The johns hopkins precursors study. *Arch. Intern. Med.* **2002**, *162*, 657–662. [CrossRef] [PubMed]
23. Winkelmayer, W.C.; Stampfer, M.J.; Willett, W.C.; Curhan, G.C. Habitual caffeine intake and the risk of hypertension in women. *JAMA* **2005**, *294*, 2330–2335. [CrossRef] [PubMed]
24. Hu, G.; Jousilahti, P.; Nissinen, A.; Bidel, S.; Antikainen, R.; Tuomilehto, J. Coffee consumption and the incidence of antihypertensive drug treatment in finnish men and women. *Am. J. Clin. Nutr.* **2007**, *86*, 457–464. [PubMed]
25. Uiterwaal, C.S.; Verschuren, W.M.; Bueno-de-Mesquita, H.B.; Ocke, M.; Geleijnse, J.M.; Boshuizen, H.C.; Peeters, P.H.; Feskens, E.J.; Grobbee, D.E. Coffee intake and incidence of hypertension. *Am. J. Clin. Nutr.* **2007**, *85*, 718–723. [PubMed]
26. Grosso, G.; Stepaniak, U.; Polak, M.; Micek, A.; Topor-Madry, R.; Stefler, D.; Szafraniec, K.; Pajak, A. Coffee consumption and risk of hypertension in the polish arm of the hapiee cohort study. *Eur. J. Clin. Nutr.* **2016**, *70*, 109–115. [CrossRef] [PubMed]
27. Rhee, J.J.; Qin, F.; Hedlin, H.K.; Chang, T.I.; Bird, C.E.; Zaslavsky, O.; Manson, J.E.; Stefanick, M.L.; Winkelmayer, W.C. Coffee and caffeine consumption and the risk of hypertension in postmenopausal women. *Am. J. Clin. Nutr.* **2016**, *103*, 210–217. [CrossRef] [PubMed]
28. Beltran-Sanchez, H.; Finch, C.E.; Crimmins, E.M. Twentieth century surge of excess adult male mortality. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 8993–8998. [CrossRef] [PubMed]

29. Yang, A.; Palmer, A.A.; de Wit, H. Genetics of caffeine consumption and responses to caffeine. *Psychopharmacology* **2010**, *211*, 245–257. [CrossRef] [PubMed]
30. Djordjevic, N.; Ghotbi, R.; Jankovic, S.; Aklillu, E. Induction of CYP1A2 by heavy coffee consumption is associated with the CYP1A2 −163C>A polymorphism. *Eur. J. Clin. Pharmacol.* **2010**, *66*, 697–703. [CrossRef] [PubMed]
31. Cornelis, M.C.; El-Sohemy, A.; Kabagambe, E.K.; Campos, H. Coffee, CYP1A2 genotype, and risk of myocardial infarction. *JAMA* **2006**, *295*, 1135–1141. [CrossRef] [PubMed]
32. Palatini, P.; Dorigatti, F.; Santonastaso, M.; Cozzio, S.; Biasion, T.; Garavelli, G.; Pessina, A.C.; Mos, L. Association between coffee consumption and risk of hypertension. *Ann. Med.* **2007**, *39*, 545–553. [CrossRef] [PubMed]
33. Bankir, L.; Perucca, J.; Norsk, P.; Bouby, N.; Damgaard, M. Relationship between sodium intake and water intake: The false and the true. *Ann. Nutr. Metab.* **2017**, *70*, 51–61. [CrossRef] [PubMed]
34. Grosso, G.; Bella, F.; Godos, J.; Sciacca, S.; Del Rio, D.; Ray, S.; Galvano, F.; Giovannucci, E.L. Possible role of diet in cancer: Systematic review and multiple meta-analyses of dietary patterns, lifestyle factors, and cancer risk. *Nutr. Rev.* **2017**, *75*, 405–419. [CrossRef]
35. Zhao, Y.; Wang, J.; Ballevre, O.; Luo, H.; Zhang, W. Antihypertensive effects and mechanisms of chlorogenic acids. *Hypertens. Res.* **2012**, *35*, 370–374. [CrossRef] [PubMed]
36. Sato, Y.; Itagaki, S.; Kurokawa, T.; Ogura, J.; Kobayashi, M.; Hirano, T.; Sugawara, M.; Iseki, K. In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. *Int. J. Pharm.* **2011**, *403*, 136–138. [CrossRef] [PubMed]
37. Suzuki, A.; Yamamoto, M.; Jokura, H.; Fujii, A.; Tokimitsu, I.; Hase, T.; Saito, I. Ferulic acid restores endothelium-dependent vasodilation in aortas of spontaneously hypertensive rats. *Am. J. Hypertens.* **2007**, *20*, 508–513. [CrossRef] [PubMed]
38. Suzuki, A.; Yamamoto, N.; Jokura, H.; Yamamoto, M.; Fujii, A.; Tokimitsu, I.; Saito, I. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. *J. Hypertens.* **2006**, *24*, 1065–1073. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Polyphenolic Nutrients in Cancer Chemoprevention and Metastasis: Role of the Epithelial-to-Mesenchymal (EMT) Pathway

Haneen Amawi [1], Charles R. Ashby Jr. [2], Temesgen Samuel [3], Ramalingam Peraman [4] and Amit K. Tiwari [1,*]

1 Department of Pharmacology and Experimental Therapeutics, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, Toledo, OH 43614, USA; haneen.amawi@rockets.utoledo.edu
2 Pharmaceutical Sciences, College of Pharmacy, St. John's University Queens, New York, NY 11432, USA; cnsratdoc@optonline.net
3 Department of Pathology, School of Veterinary Medicine, Tuskegee University, Tuskegee, AL 36088, USA; tsamuel@mytu.tuskegee.edu
4 Medicinal chemistry Division, Raghavendra Institute of Pharmaceutical education and Research (RIPER)-Autonomous, Anantapur 515721, India; drramalingamp@gmail.com
* Correspondence: amit.tiwari@utoledo.edu; Tel.: +1-419-343-1913

Received: 12 July 2017; Accepted: 11 August 2017; Published: 21 August 2017

Abstract: The epithelial-to-mesenchymal transition (EMT) has received significant interest as a novel target in cancer prevention, metastasis, and resistance. The conversion of cells from an epithelial, adhesive state to a mesenchymal, motile state is one of the key events in the development of cancer metastasis. Polyphenols have been reported to be efficacious in the prevention of cancer and reversing cancer progression. Recently, the antimetastatic efficacy of polyphenols has been reported, thereby expanding the potential use of these compounds beyond chemoprevention. Polyphenols may affect EMT pathways, which are involved in cancer metastasis; for example, polyphenols increase the levels of epithelial markers, but downregulate the mesenchymal markers. Polyphenols also alter the level of expression and functionality of important proteins in other signaling pathways that control cellular mesenchymal characteristics. However, the specific proteins that are directly affected by polyphenols in these signaling pathways remain to be elucidated. The aim of this review is to analyze current evidence regarding the role of polyphenols in attenuating EMT-mediated cancer progression and metastasis. We also discuss the role of the most important polyphenol subclasses and members of the polyphenols in reversing metastasis and targeting EMT. Finally, limitations and future directions to improve our understanding in this field are discussed.

Keywords: polyphenols; chemoprevention; cancer; metastasis; epithelial mesenchymal transition

1. Introduction

The consumption of fruits and vegetables rich in certain polyphenols has been reported to decrease the incidence and mortality of cancer, as well as delaying cancer progression [1]. The adverse effects and toxicities of many of the currently chemotherapeutic drugs have spurred research with certain phytochemicals for chemoprevention and treatment of cancer [2,3]. Polyphenols are natural phytochemicals that are present in high amounts in many plants (including plant seasonings), fruits, vegetables, seeds, oils, and alcoholic and non-alcoholic beverages [4]. Indeed, polyphenols have been shown to inhibit the proliferation of cancers of the mouth, gastrointestinal tract, liver, lung, breast and skin both *in vivo* and *in vitro* [5]. However, the molecular mechanisms that mediate the chemopreventive efficacy of the polyphenols remain to be elucidated to a large extent. Furthermore, there are considerable discrepancies between the health benefits versus the clinical outcomes with

the intake of polyphenol. This could be due to a number of factors, with one of the major ones being the testing of non-physiological concentrations of polyphenols, thereby potentially obscuring the mechanism of action at therapeutic doses. Current meta-analyses of observational studies showed limited evidence of a correlation between dietary polyphenol intake and cancer risk, with most of the significant findings related to a decreased risk of lung, stomach, breast, and colorectal cancers [6]. Nonetheless, accumulating evidence suggests that epithelial-mesenchymal transition (EMT), one of the main pathways involved in cancer development and metastasis, is modulated by a number of polyphenolic compounds [7,8]. During EMT, cells transition from an epithelial to a mesenchymal state and lose their cell–cell adhesions, cell polarity and differentiation properties [9]. These changes induce the cells to become motile and invasive, allowing migration through the extracellular matrix, reaching distant tissues [10]. Numerous polyphenolic compounds, including, but not limited to, flavonoids, ellagic acid, quercetin, silymarins, resveratrol and curcumin, have been reported to significantly reverse metastasis and invasiveness in different cancers *in vitro* and *in vivo*. Therefore, it is possible that polyphenolic compounds may reverse or prevent cancer progression, invasion and metastasis by inhibiting the EMT signaling pathways in cancer cells. However, most of the reported benefits of polyphenols were obtained from preclinical studies in cells or animals where the doses used were significantly higher than the amount usually obtained from the consumption of food [11]. The exact intake and bioavailability of polyphenols are still unknown, greatly restricting their clinical use for cancer chemoprevention, treatment and reversal of metastasis.

To the best of our knowledge, only a few previous comprehensive reviews have been published describing the effects of different phytochemicals on EMT and metastasis [7,8]. Our review specifically describes the effects of polyphenols on EMT signaling and related proteins in preventing and treating cancer metastasis. The goal of this review is to provide the reader with a better understanding of the interactions between natural polyphenols and the inhibition of EMT signaling and chemoprevention for cancer development and metastasis. This review will present some of the complex molecular mechanisms that may be involved in mediating the chemo-preventative efficacy of the natural polyphenols.

2. Dietary Polyphenols and Health Benefits

Polyphenols are one of the most abundant phytochemicals in plants and have the highest intake by humans compared to other natural products [12]. Fruits, vegetables, herbs, cereals, tea, coffee, nuts, seeds and beer are rich sources of polyphenols [13]. Polyphenolic compounds are secondary metabolites that accumulate in the plant leaves and flowers to protect plants against diseases, infections and damage [14–16]. The structures of the family members are highly diverse and complex, with molecular weights ranging between 500 and 3000 Da [17]. Therefore, depending on the number of phenolic rings and the interconnection of these rings, the polyphenolic family is divided into several major classes, with thousands of members in each class, including flavonoids, phenolic acids, stilbenes, and lignans (Figure 1) [13]. Coumarins are also considered as a separate subgroup of polyphenols that range from simple to polycyclic coumarins [18,19]. Flavonoids are the largest class in the group and can be categorized as flavonols (e.g., quercetin, kaempferol) [20], flavones (e.g., apigenin, luteolin) [21], flavanones (e.g., naringenin and hesperetin) [22], isoflavones (e.g., genistein and daidzein) [23,24], flavanols (e.g., catechin, epigallocatechinepigallocatechin gallate) [18,25], anthocyanins (e.g., cyandin, malvidin) [26], and flavonolignans. Phenolic acids are the second most common class of polyphenols after flavonoids and are present in coffee and black tea [27]. They are primarily classified as benzoic and cinnamic acid derivatives [28]. Stilbenes are not common in plants and are only produced upon pathogen invasion [29]. Finally, lignans are phytoestrogens that are highly abundant in flaxseed and flaxseed oil, with the best-known compounds being secoisolariciresinol and matairesinol [30]. The structure of polyphenols is an important determinant of their bioavailability, pharmacokinetic profile, interactions with biomolecules and efficacy [30].

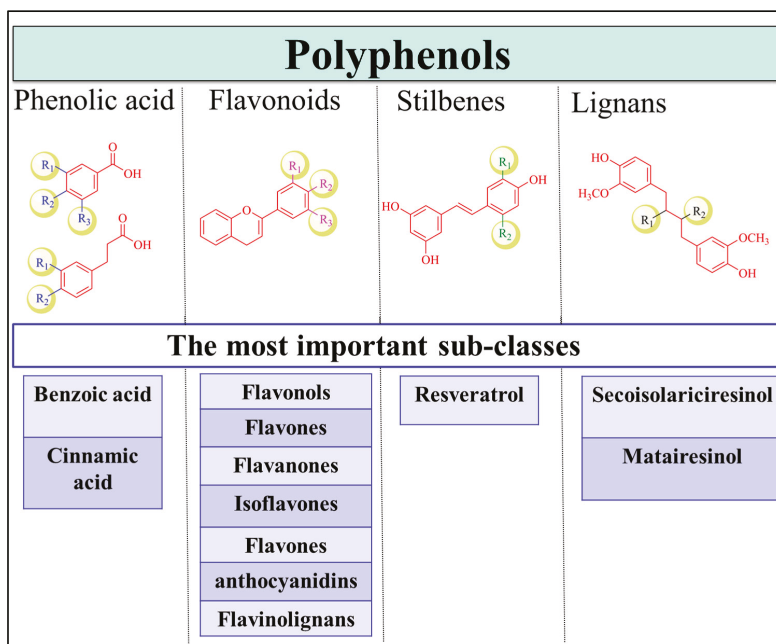


Figure 1. Polyphenol classification. The classes of polyphenols include phenolic acids, flavonoids, stilbenes, and lignans. Examples of important subfamilies of each class are shown.

Polyphenols are reducing compounds that have been reported to prevent inflammation [31], oxidative stress [12], cardiovascular disease [32], infections [13,33], bone diseases [34] and cancer [35], among others [36]. They can form complexes with certain proteins, scavenge free radicals and modulate several signaling pathways [37,38]. However, the diversity of the distribution of dietary polyphenols in plants, complexation with other plant constituents, degradation of these compounds during food processing, and their poor pharmacokinetic profile due to their complex structures and interactions with gut microflora and other biomolecules limit the efficacy of polyphenols [30]. In addition, the exact determination of the daily intake of dietary polyphenols is still uncertain and is subject to significant variation between different individuals. Moreover, the diversity of the structures in this family makes bioavailability a function of the individual polyphenols. Generally, after oral ingestion, polyphenols are present in low concentration in the blood and urine, as they interact with gut microflora, are primarily metabolized by methylation, glucuronidation and sulfation, and are rapidly excreted in the bile and urine [39]. However, the metabolites of certain polyphenolic compounds can significantly contribute to the biological activity or efficacy of the parent polyphenolic compounds [40]. Clearly, additional research is required to improve our knowledge about the intake and bioavailability of dietary polyphenols to determine their efficacy in disease prevention.

Polyphenols in Cancer Prevention and Treatment

Cancer is an amalgam of complex, heterogeneous diseases, in which many signaling pathways are affected [41]. The processes of initiation, promotion and progression are required for the development and invasiveness of cancer [42]. In cancer cells, there is a significant disruption of the cell cycle, uncontrolled cell proliferation, and dysregulation of processes that produce cell death, such as apoptosis [43]. Dietary factors, especially those from plants, have been reported to reduce the risk of various cancers and malignancies [44]. According to the American Cancer

Society (cancer facts and figures, 2017), about 20% of cancers in the United States can be prevented by a healthy lifestyle, including a diet rich in vegetable and fruits and physical activities [45]. Polyphenol-rich foods, based on preclinical, clinical and epidemiological studies, have been reported to have chemopreventive and anticancer efficacy [46–48]. Polyphenolic compounds can inhibit the proliferation of prostate, bladder, lung, gastrointestinal, breast and ovarian cancers [49]. Quercetin, resveratrol, green tea polyphenols [50], epigallocatechin-3-gallate [51] and curcumin [52] have efficacy as anticancer compounds. It has been hypothesized that polyphenols may prevent (1) cancer initiation (cytoprotective); (2) relapse; or (3) its progression and metastasis to distant organs (cytotoxic) [53–55]. The cytoprotective (i.e., chemopreventive) effect of the polyphenols is attributed primarily to their antioxidant activities [56]. However, the actual anticancer efficacies of polyphenols are due to antioxidant-independent mechanisms, including their pro-oxidant action [49,56]. Therefore, polyphenols may produce antioxidant effects in normal cells, while inducing pro-oxidant damage in cancer cells.

Previously, it was postulated that the chemopreventive efficacy of polyphenols is primarily due to their antioxidant action [5]. Polyphenolic compounds have hydroxyl groups that donate their protons to a reactive oxygen species (ROS) [44]. In addition to their antioxidant effects, polyphenols inhibit the activity of phase I enzymes, primarily cytochrome P450 enzymes (CYPs), such as CYP1A1 and CYP1B1 [57]. This prevents the formation of reactive and carcinogenic metabolites [57]. The polyphenols also induce phase II enzymes, which increase the formation of polar metabolites that are readily excreted from body [58]; for example, dietary polyphenols inhibit the development of lung cancer and exert significant chemopreventive effects *in vitro* and *in vivo* [44]. Furthermore, it has been reported that certain dietary polyphenols decrease the cellular formation of ROS, which decreases the oxidation of DNA, proteins and lipids [44].

However, recent data suggests that the pro-oxidant, not the antioxidant, properties of the polyphenols may be important in treating and preventing cancer. The pro-oxidant activities of polyphenols in cancer cells generates ROS, producing [49,59,60] (1) cell cycle arrest [61]; (2) induction of apoptosis and DNA fragmentation [62]; (3) inhibition of proliferation signaling pathways, including epidermal growth factor receptor/mitogen activated protein kinase (EGFR/MAPK), phosphatidylinositide 3-kinases/protein kinase B (PI3K/Akt) [63], and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [64] and (4) anti-inflammatory effects [65,66]. For example, the polyphenols, at 0.3 mg/ml, from apples, inhibit human bladder transitional cell carcinoma (TCC, TSGH-8301 cells) proliferation, induce G2/M cell cycle arrest, and promote apoptosis and mitotic catastrophe [67]. Green tea polyphenols (e.g., pigallocatechin gallate and black tea theaflavins), in human papilloma virus-18-positive HeLa cervical cancer cells induced death, cell cycle arrest at the subG1 phase, apoptosis through caspases activation, and ROS generation. These aforementioned effects were mediated by inhibiting Akt and NF-κB signaling in these cells [68].

Flavonoids (e.g., apigenin, quercetin, luteolin, fisetin) were reported to induce apoptosis in cancer cells, including leukemic U937 cells [69], prostate cancer cells [70], hepatic cancer cells [71], and others. Curcumin is a polyphenol extracted from *Curcuma longa* (tumeric) [72]. Its anticancer efficacy was shown to be mediated through different mechanisms, including TNF-induced apoptosis, the inhibition of NF-κB, and the inhibition of Wnt/β-catenin and EMT signaling in breast, colon, brain and other cancers [73]. However, as mentioned earlier, the efficacy of the polyphenols is limited by their less than optimal pharmacokinetic profile [74]. Certain polyphenols lack significant efficacy due to their limited bioavailability, the amount consumed and the cancer type and stage [75,76]. Accordingly, the diversity in the response to polyphenols is dependent upon the dose, cancer cell type, and the patient's genome [42].

3. Polyphenols Role in Reversing EMT Mediated Cancer Metastasis

3.1. Metastasis

The metastasis of cancer consists of at least five phases and ultimately >90% of relapsed cancer patients die from metastasis [77]. A significant proportion (at least 70%) of cancer patients has metastasis following their initial cancer diagnosis [78]. In metastasis, the cancer cells degrade the basement membrane, invade the surrounding vasculature, enter into the blood and/or lymph circulation, migrate to the distant tissues and organs, and finally implant, colonize and proliferate to form new tumor masses [79]. The proposed molecular mechanisms involved in the development of cancer metastasis are very complex and numerous, involving different, unrelated genes [80,81]. This complexity interferes with the prediction of the probability of the development of metastasis, which affects the diagnosis and treatment of metastatic disease [78]. The tumor cells adopt new phenotypes, characterized by the overexpression of certain genes associated with metastasis, in combination with the downregulation of genes that when transcribed, produce proteins that inhibit metastasis [82]. Numerous studies have reported the differential expression of genes during metastasis, where numerous signaling pathways, including EMT, are dysregulated [83–87]. The level of metastasis is dependent upon a number of variables, including, but not limited to, the type of cancer, cancer stage, patient genetic profile and mutations and gender [88,89]. Currently, there is no clinically approved treatment that specifically targets metastasis. Typically, metastasis is treated using conventional therapies, such as radiotherapy, chemotherapy and surgery, which are used to treat primary tumors. The goal of the treatment for metastatic cancer is to control the metastasis and improve patient survival, as opposed to curing the disease [90]. Thus, it is imperative to find treatments that target molecules involved in the development and maintenance of metastasis.

3.2. EMT Role in Mediating Cancer Metastasis

Although EMT is essential for the developmental formation of organs and tissue repair, its pathological activation can lead to an increased risk of cancer and fibrosis [9]. Accumulating data suggests that the epithelial-mesenchymal transition (EMT) is one of the primary and early pathways involved in cancer development and metastasis [10,91,92]. In the early stages of metastasis, EMT activates cells to transition from an epithelial to a mesenchymal state where cells lose their cell–cell adhesions, cell polarity and differentiation properties (Figure 2). These changes decrease the probability of apoptosis and increase cell motility and tissue invasiveness, allowing them to migrate through the extracellular matrix, reaching distant tissues (Figure 2) [10]. On a morphological level, the cells lose their round shape and become spindle-shaped, which is associated with an increase in mobility and invasiveness [92]. This occurs, in part, from the loss of epithelial cell adhesion proteins, (e.g., E-cadherin) and the upregulation of the expression of mesenchymal proteins (e.g., N-cadherin, vimentin) [93]. E-cadherin is a transmembrane glycoprotein that is typically highly expressed on differentiated epithelial cells, where it regulates cell adhesion [94]. The adhesion process involves the formation of interactions between E-cadherin, located at the surface of the neighbor cells [95].

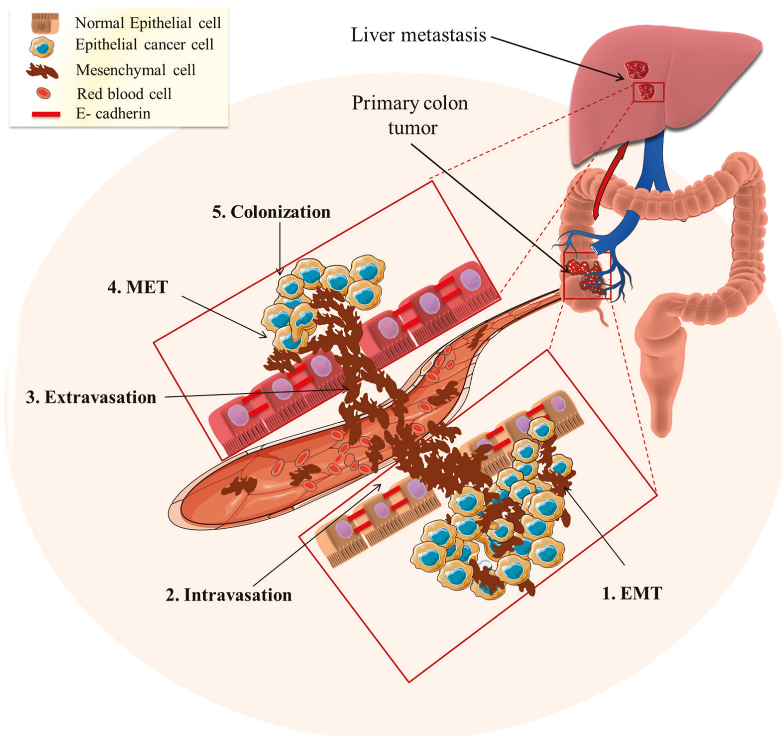


Figure 2. Epithelial to mesenchymal transition (EMT) role in cancer metastasis. The tumor epithelial cells transform mesenchymal invasive cells through EMT (1. EMT). Subsequently, the mesenchymal cells enter the blood circulation to distant places (2. Intravasation) and this results in the homing of circulating tumor cells to specific organs or tissues (3. Extravasation). The metastasized mesenchymal cells transition to the epithelial phenotype through the mesenchymal–epithelial transition (4. MET). The MET-transformed cancer cells become implanted and interact to form new colonies (5. Colonization) and ultimately forming a tumor.

The loss of E-cadherin expression is a hallmark and direct event required for the development of EMT, which is positively correlated to the development of metastatic cancers [94]. Alterations in various signaling pathways have been shown to induce the loss of E-cadherin and facilitate the transition from epithelial to mesenchymal state. For example, EMT can be regulated by extracellular signal-regulated kinases (ERK1/2) [96], tyrosine kinases [97], transforming growth factor β (TGFβ) [98], insulin like growth factor (ILGF) [99], epidermal growth factor (EGF) [100], platelet derived growth factor (PDGF) [101], NF-κB [102], protein kinase B (Akt) [103] and the (Wnt)/β-catenin signaling pathway (Figure 3) [104]. All the above proteins can activate nuclear transcriptional factors, such as snail, twist and zinc finger E-box binding homeobox (ZEB1/2), which directly bind to the E-cadherin gene promoter region, suppressing gene expression [105–108]. In addition, mutations in oncogenes and suppressor genes, DNA methylation and changes in microRNAs are also involved in the induction process [108]. In contrast to EMT, the mesenchymal–epithelial transition (MET) is the transition of mesenchymal cells to an epithelial phenotype [109]. The presence of EMT and MET provides cancer cells with flexibility, where initially they use EMT to detach, pass through the basement membrane and reach the distant tissues. Subsequently, the cells can adopt the epithelial phenotype again, leading to the development of metastasis [10].

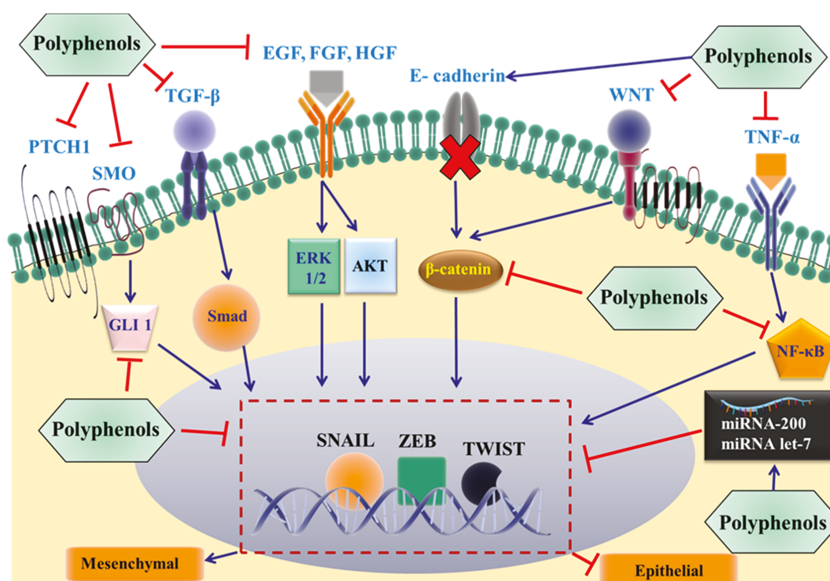


Figure 3. Proposed mechanisms by which dietary polyphenols inhibit EMT and cancer metastasis. The most important extracellular signals are shown, and these promote the epithelial to mesenchymal transition by binding to individual membrane receptors, eventually activating specific EMT-inducing transcription factors (snail, Zeb, and/or twist). The effect of the polyphenols on specific proteins in the signaling pathways at different levels remains to be elucidated. TGF-β: transforming growth factor β; EGF: epidermal growth factor; HGF: hepatocyte growth factor; FGF: fibroblast growth factor; PTCH1: Patched 1; SMO: smoothened; WNT: glycoprotein family; TNF-α: Tumor necrosis factor-α; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; GLI 1: glioma-associated oncogene 1; ERK: extracellular regulated protein kinases; AKT: protein kinase B.

Wnt/β-catenin signaling and EMT are closely related and are involved in the positive feedback activation of one another [110]. Wnt/β-catenin signaling is a crucial pathway in cell–cell communication, where one cell releases the Wnt protein to bind to an adjacent cell's surface receptor [111]. This binding results in the initiation of cellular signals that are transmitted to the nucleus to induce genetic modifications [112]. β-catenin is the key regulatory protein for signal transmission in Wnt signaling pathways [111]. It is also involved in structural and cell adhesion functions, where it connects the membrane-associated E-cadherin to cellular actin [95]. Upon activation by Wnt, cytosolic β-catenin is stabilized and translocated from the cytoplasm to nucleus, interacting with DNA binding proteins, such as lymphocyte enhancer factor and T cell factor complex (TCF/EF1), inducing the transcription of several genes (e.g., T-cell factor 1 (TCF1), matrix metalloproteinase-7 (MMP-7), the cluster of differentiation 4 (CD4) protein and cyclin D1) [113]. Finally, these genes regulate cellular proliferation, development, survival and metastasis [114]. In the absence of the extracellular Wnt, β-catenin translocation to the nucleus is inhibited by the β-catenin destruction complex, which consists of adenomatous polyposis coli (APC), axin, glycogen synthase kinase (GSK3β) and casein kinase 1 (CK1α)) [115,116].

E-cadherin binds directly to β-catenin, where the conserved carboxyl terminus of E-cadherin binds to the APC binding site of β-catenin in the cytoplasm and interacts with actin microfilaments [117]. This binding is essential for mediating cadherin-based cell adhesion with cytoskeletal actin. A decrease in the expression of E-cadherin is positively correlated with an increased activation of Wnt/β-catenin signaling, induction of EMT, and rapid progression of cancer [118,119]. Another interaction between

the two pathways involves the zinc finger transcriptional receptor, snail. It binds to the promoter region of the E-cadherin gene, mediating the repression of E-cadherin gene expression [120]. The repression of E-cadherin is positively correlated with the upregulation of Wnt/β-catenin signaling [121]. Snail and β-catenin interact physically and functionally, exerting a positive feedback effect on one another [122]. The pooling or accumulation of snail in the cytoplasm is regulated by the GSK3β-induced phosphorylation of snail [122,123]. Thus, Wnt signaling can stabilize and increase the levels of snail in the cytoplasm by the inhibition of GSK3β [123]. Both snail and β-catenin synergize and stabilize each other's biological effects and transcriptionally augment E-cadherin-induced repression on epithelial cells and facilitate EMT in cancer [124].

Recently, EMT has been identified and recognized as one of the factors contributing to the development of multidrug resistance (MDR) in cancer cells [125]. Several studies indicate that different cancer cells expressing MDR phenotypes also typically express the EMT phenotype [126]. These MDR cells express higher levels of N-cadherin and vimentin, but express lower levels of E-cadherin and cell-cell junctions, suggesting that EMT is positively correlated with the MDR phenotype [125,127]. In addition, one study has shown that cells undergoing EMT have a significantly lower response to the chemotherapeutic drug, cyclophosphamide, compared to cells not undergoing EMT [128]. Consequently, MDR could be targeted by developing drugs that also inhibit pathologically-induced EMT [127,128]. Finally, inhibiting EMT could be a promising strategy to limit cancer cell diffusion and stemness, which could lower the rate of mortality in patients with metastatic cancer.

3.3. Polyphenols Effectiveness in Reversing Metastasis

The initial studies with polyphenols were predominantly focused on reducing cancer risk by dietary polyphenols, as well as the treatment of primary tumors [129]. However, over the past decade, there has been a significant increase in determining the efficacy of polyphenols in metastatic cancer [130]. Indeed, dietary polyphenols, such as epicatechin, epigallocatechin (EGC), delphinidin tannins, epigallocatechin-3-gallate (EGCG), green tea catechins, quercetin and luteolin among others, inhibit cell wound healing and transwell migration *in vitro* in several cancer cell lines [131,132]. Carnosol, a dietary diterpene, has been reported to inhibit the viability of human breast, ovarian and intestinal tumor cell lines [133]. The combination of carnosol with curcumin also significantly inhibited the viability of primary cancer cells isolated from the pleural fluid or ascites of patients with metastatic cancers [133]. The antimetastatic efficacy of certain polyphenols was also reported *in vivo*, where polyphenols inhibited the development of metastasis and significantly improved the survival of animals in metastatic models [134]. The mechanisms by which these compounds produce antimetastatic efficacy are complex. For example, polyphenols significantly downregulate the expression of matrix metalloproteinases, such as MMP-2 and MMP-9, which promote cellular invasion [135,136]. The hepatocyte growth factor receptor (HGFR or MET) activates epithelial cell dissociation and invasive branching, is also significantly inhibited by several polyphenols, including (−)-Epigallocatechin-3-gallate (EGCG) [137,138]. Polyphenols significantly reduce the activation and the nuclear translocation of NF-κB, further inhibiting the signaling pathways and affecting certain genes [139,140]. The formation of new blood vessels, known as angiogenesis, is considered to be a primary mechanism for metastatic cells to reach the blood and migrate to new sites in the body [141]. Polyphenols have been reported to interfere with vascular endothelial growth factor (VEGF)-mediated angiogenesis [142]. Finally, polyphenols also decrease EMT signaling and reverse metastasis by altering the levels of proteins involved in this pathway.

3.4. Polyphenols Reverse Metastasis by Targeting EMT

As discussed above, polyphenols, in part, produce their antimetastatic efficacy by targeting the EMT pathways. Currently, only a few studies have determined the effects of whole plant polyphenols extract. The majority of the studies were focused on a certain class or specific polyphenolic compounds. Consequently, this does not allow researchers to determine the effect of whole plant polyphenol

extracts on EMT and related pathways. There are data suggesting that whole polyphenol extracts can reverse metastasis and modulate EMT. For example, the extracted polyphenols from *A. annua* L. (pKAL), including hydroxycinnamic acids and flavonoids, were evaluated in metastatic breast cancer cells (MDAMB-231) [143]. The compound pKAL significantly inhibited the TNF-α-induced migration and invasion of MDAMB-231 cells (1–30 μg/mL), with minimal toxicity in normal cells. Furthermore, pKAL caused cells to become a more rounded, epithelial-like phenotype [143]. pKAL also significantly inhibited the mesenchymal markers N-cadherin and snail, but not E-cadherin or β-catenin. Thus, the modulation of EMT could play a role in the anti-metastatic mechanism of action in MDAMB-231 cells [143]. In addition, the anti-metastatic efficacy of the polyphenol mixtures, mainly quercetin and kaempferol from *Euphorbia supina* (PES), was determined in metastatic breast cancer MDA-MB-231 cells [46]. PES significantly inhibited the expression levels of N-cadherin, snail and MMP-9 at concentrations up to 5 μg/mL [144]. Curcumin (20–40 μM) significantly inhibited the Wnt/β-catenin-mediated activation of EMT, where β-catenin, vimentin, and TCF4 were significantly downregulated and E-cadherin was significantly upregulated in SW620 cells [145]. These findings support the hypothesis that polyphenols may produce their chemopreventive and antimetastatic efficacy primarily by modulating EMT and its network of proteins. The role of the most important classes of polyphenols and their members in the prevention and reversal of EMT-mediated cancer metastasis is discussed below in detail.

3.4.1. Flavonoids

Flavonoids are the most abundant dietary polyphenols in fruits, vegetables, flowers, chocolate, tea, wine and other edible plants [74]. The basic chemical structure for all members in the flavonoid family is two benzene rings, connected by a 3-carbon bridge, forming a heterocycle (i.e., C6–C3–C6) [146]. The anticancer and antimetastatic efficacy of the flavonoids has been investigated extensively and confirmed in both *in vitro* and *in vivo* models [147]. However, the exact mechanisms by which these compounds produce their efficacy remains to be elucidated, as with other polyphenols [148]. However, there is robust evidence that modulation of EMT and its related signaling pathways is one of the primary determinants of their efficacy [149]. For example, epigallocathechin gallate (EGCG), a flavan-3-ol, induced apoptosis (40 μM), significantly inhibited colony formation and cell migration (20 and 40 μM) in nasopharyngeal carcinoma (NPC) cancer stem cells (CSC), including the sphere-derived NPC TW01 and TW06 cell lines [150]. Interestingly, EGCG significantly upregulated the expression of the epithelial marker, E-cadherin, and downregulated the expression of snail and vimentin at 20 and 40 μM [150]. The incubation of the invasive and metastatic A431-III cell line with 20 μM luteolin and quercetin, for 48 h, significantly suppressed the expression of the following mesenchymal markers and transcriptional factors: Fibronectin, vimentin, twist, snail, and N-cadherin and relocalized E-cadherin adhesions on the cell membrane, yielding a more epithelial-like phenotype [151]. In addition, 20 μM of luteolin and quercetin reversed the migration and invasiveness of the A431-III cells and downregulated MMP-9 expression, which significantly induces EMT and cell invasion [151].

3.4.2. Stilbenes

The stilbene polyphenolic compounds are characterized by a di-methylene bridge connecting the two phenolic rings [152]. Resveratrol is the most well-known of stilbenes and has been reported to significantly inhibit the metastasis of various cancers [136,153,154]. Resveratrol significantly inhibits the metastasis of colon cancer cell line (LOVO) both *in vitro* and *in vivo* (significantly at >50 mg/kg) [153]. Resveratrol also significantly inhibited gastric cancer cell (SGC-7901) metastasis *in vitro* and reversed both hedgehog and EMT signaling at ≈100 μM [155]. The glioma-associated oncogene 1 (Gli-1), N-cadherin and snail were significantly downregulated, in tandem with a subsequent significant upregulation in E-cadherin levels [155]. Resveratrol (6–60 μM) induced the presence of epithelial characteristics in cancer cells following their exposure to compounds that induce EMT [154,156].

For example, resveratrol, at 6 μM and 12 μM, significantly inhibited TGF-β1-induced (10 ng/mL for 48 h) EMT in LOVO cells by upregulation of E-cadherin and downregulation of vimentin [153]. Similarly, epidermal growth factor (100 ng/ml) induced EMT in MCF-7 breast cancer cells, and this was prevented and reversed by 25 μM of resveratrol, which, in turn, inhibited EMT [154]. Resveratrol, at 25 μM, inhibited wound healing and cell motility, significantly downregulated vimentin and N-cadherin, and significantly upregulated E-cadherin levels in MCF-7 cells [154]. Resveratrol (25 μM) also significantly downregulated EMT-inducing transcriptional factors, such as ZEB 1/2 and snail [154]. Similar results were obtained in ovarian cancer cells, where resveratrol (20–60 μM) significantly inhibited cisplatin-induced EMT [156]. Finally, novel analogues of resveratrol have been reported to enhance the inhibition of the proliferation and migration of ovarian cancer cells, compared to resveratrol [157]. These analogues also inhibited the expression of epithelial to mesenchymal transition (EMT) markers [157]. Overall, these results indicate that resveratrol exerts a significant part of its anti-metastatic efficacy through altering the EMT pathway.

3.4.3. Phenolic Acids

The phenolic acid class of polyphenols is the second most abundant group in the family, accounting for 30% of the dietary polyphenols [158]. The phenolic compounds are divided into either hydroxybenzoic acid compounds, such as gallic acid or hydroxycinnamic acid, such as caffeic acid and ferulic acid [159,160]. These members of the phenolic family were evaluated for anticancer and antimetastatic efficacy and their effects on the mesenchymal characteristics of cancer cells [161,162]. Caffeic acid, ellagic acid, gallic acid and ferulic acid inhibit cancer cell proliferation and metastasis in different cancer models [161,163–165]. Furthermore, anacardic acid suppresses prostate cancer angiogenesis and migration alone or in combination with radiation by different mechanisms [166,167]. In primary human umbilical vascular endothelial cells (HUVECs), anacardic acid (20 μM) inhibited Src and focal adhesion kinase (FAK), activation of RhoA-GTPase and inactivation of Rac1 and Cdc42-GTPases [167]. Phenolic compounds have also been reported to reverse metastasis induced by EMT. For example, caffeic acid (50–100 μM for 48 h) significantly inhibited the migratory capability and reversed EMT to MET (epithelial and adhesive phenotype) in the skin cancer cell line, HaCaT [168]. Additionally, caffeic acid (100 μM) significantly inhibited the activation of the NF-κB/snail signal pathway, a strong inducer of EMT [168]. Furthermore, E-cadherin expression was significantly increased, whereas N-cadherin and vimentin levels were significantly downregulated by caffeic acid (100 μM) [168]. Ferulic acid significantly inhibits EMT-induced metastasis of MDAMB-231 cells *in vitro* (10–30 μM) and in an *in vivo* mouse xenograft model (100 mg/kg/day) [165]. Ferulic acid (200 μM) significantly blocked P-Smad2/3 activation and attenuated all of the EMT changes induced by 5 ng/ml of TGF-β1 in rat kidney epithelial cells (NRK-52E) [169]. Furthermore, ferulic acid (200 μM) significantly upregulated E-cadherin and downregulated fibronectin, snail, and integrin linked kinase (ILK) in the same cell line [169]. Although the direct effect of other phenolic compounds was not investigated directly on EMT markers, such as N-cadherin or vimentin, they modulated other proteins that significantly affect EMT. Proteins, such as MMP-9, TGF-β, IL-6, β-catenin, NFκB, VEGF, and other oncogenic signaling proteins, were significantly downregulated by caffeic acid, ellagic acid, gallic acid and other phenolic acid compounds at 50–200 μM [66,161,169–172]. Ellagic acid, when combined with luteolin and punicic acid, at 64 μg/component/day, significantly inhibited primary prostate cancer growth, prevented metastasis, and significantly decreased VEGF and IL6 levels *in vivo* [172]. Gallic acid significantly inhibited the migration and invasion of A375.s2 human melanoma cells by inhibiting the zinc-dependent, proteolytic enzyme MMP-2 [173].

3.4.4. Lignans

The dimerization of two cinnamic acids results in a 2,3-dibenzylbutane structure, forming the lignans class in the polyphenolic family [174]. Secoisolariciresinol and matairesinol are the two most well-known lignans [175]. Lignans have been shown to have chemopreventitive efficacy in breast

and prostate cancers in humans [176–178]. Flaxseed supplementation of secoisolariciresinol (SDG) at 73, 147 or 293 µmol/kg, which is equivalent to 2.5%, 5% or 10% flaxseed diet, significantly reduced metastasis in a melanoma mice model where the percent of animals that had >50 lung tumors was reduced to 30%, 21%, and 22%, respectively, compared to the control (59%) [178]. In addition, a 10% flaxseed diet, given as 0.2 g/kg secoisolariciresinol diglycoside (SDG) and 36.53 g/kg flaxseed oil (FO), significantly reduced the growth and metastasis of estrogen receptor positive breast cancer, in part due to the presence of lignan [179]. However, to date, there have been no published reports correlating the antimetastatic potential of lignans to EMT modulation. Further studies are required to the effect of polyphenolic lignans on EMT and its interlinked pathways.

4. Future Directions

4.1. Effects of Polyphenols on Specific Proteins in the EMT Pathway

Currently, it is unknown as to what specific proteins in the EMT pathway or related pathways are affected by the polyphenols. It remains to be determined if polyphenols are altering the EMT pathways directly or act through the higher level signaling pathways or via a combination of multiple targets. A summary of the general effects of important polyphenols on EMT markers and related proteins are listed in Table 1. The direct effect of polyphenols on specific targets can be achieved by studying the EMT and related signaling pathways at the level of gene expression. For example, investigating the impact of dietary polyphenols on the expression levels and activities of microRNAs that control the mesenchymal properties of epithelial cells has not been thoroughly evaluated. Recently, the correlation of microRNA with cancer is gaining significant interest [176]. Such research would provide a better understanding and new perspectives in defining the exact targets for the efficacy of polyphenols and their exact relationship with EMT in cancer metastasis.

Table 1. A summary of the effect of selected polyphenolic compounds on the EMT markers and proteins in the related pathways.

Polyphenol subfamily	Member		Effect on EMT markers or related proteins	Studies type
Flavonoids	EGCG		↑ E-cadhrin, ↓ Veminten , Snail	*In vitro*
	Quercetin		↑ E- cadherin ↓ Fibronectin, Vimentin, Twist, Snail, MMP-9, and N-cadherin	*In vitro*
Stilbines	Resveratrol		↑ E-cadherin ↓ N-cadherin and Snail, Vimentin, ZEB 1/2, and TGF-β,	*In vitro*
Phenolic acids	hydroxybenzoic acid	gallic acid	↓ MMP-9, TGF-β, IL-6, β-catenin, NFκB, VEGF	*In vitro*
	hydroxycinnamic acid	caffeic acid	↑ E-cadherin ↓ NF-κB/snail, N-cadherin and Vimentin	*In vitro*
		Ferulic	↑ E-cadherin ↓ P-Smad2/3, Fibronectin, Snail	*In vitro* & *in vivo*
Lignans	Secoisolariciresinol		No data corelate their effect to EMT	
	Matairesinol			

Studying the Impact of Polyphenols on EMT-Related miRNAs

MicroRNAs (miRNA) are a specific type of single stranded RNA sequences that do not code for proteins. These sequences are usually short in length, consisting of only a few nucleotides (typically 19–25) and they negatively regulate their targets [180,181]. miRNA act at the mRNA level, where they complementary interact with their target mRNA. However, their binding sites lack strict specificity, so each miRNA can bind and target several mRNA, affecting apoptosis [182], disease progression [183], cancer development and metastasis [184,185]. The miRNAs are initially expressed from their genes by RNA polymerase II as Pri-miRNA. Subsequently, they become either pre-miRNA in the nucleus or mature miRNA in the cytoplasm through cleavage of different sites in their structures [186]. Recent reports indicate that more than 21 thousand mature miRNA molecules have been isolated in many different species [187].

In cancer, miRNAs are located mostly in cancer-related genomic areas, where miRNA can be either oncogenes or tumor suppressors [188]. Tumor suppressor miRNAs repress the oncogenes that induce tumor proliferation, resistance and metastasis; for example, miRNA-15 and 16 are strong inhibitors of the anti-apoptotic protein Bcl-2 [189]. Many miRNAs are significantly altered in different cancers, including breast [190], colorectal [191], brain, and others [192].

In contrast, the role of miRNA families in EMT development is diverse and controversial. Whereas some miRNAs significantly inhibited adoption of the mesenchymal phenotype in epithelial cells, other miRNAs were involved in the induction and maintenance of EMT [193]. miRNA 200 and miRNA 205 are strong suppressors of EMT and promotors of MET phenotype and produce their effects by directly binding to the promotor region of EMT, inducing transcriptional factors (e.g., snail, ZEB1/2 and twist) [194]. The ectopic overexpression of miRNA200 significantly induced higher E-cadherin levels and significantly reduced the levels of ZEB1/2 [194]. There is a significant reduction in the expression of the miRNA 200 subfamilies in metastatic breast cancer, in tandem with increased expression of the ZEB1/2 [195]. miRNA-30a significantly silenced snail transcriptional factor, inducing significant inhibition of EMT in lung carcinoma [196]. Given that EMT can facilitate cell detachment and motility, and thus cell metastasis, EMT-related miRNAs significantly affect the progression of metastasis [197]. Furthermore, some miRNA subfamilies, such as miRNA-9 [198], miRNA-103/107 [199] and miRNA-155 [200], promote cancer metastasis through the induction of EMT. Indeed, miRNA-9 enhanced the invasive and metastatic properties in breast cancer cell lines by directly binding and inhibiting the transcription of the E-cadherin gene (CDH1) miRNA-103/107 significantly suppressed the inhibition of the inhibitory effect of miRNA-200 by silencing its gene expression [201].

It is possible that the polyphenols produce their beneficial effects by altering the expression levels of several miRNAs [202]. However, only a few studies have investigated the relationship between polyphenols and miRNAs, particularly the reversal of cancer metastasis. One study showed that dietary polyphenols can alter the expression of several miRNAs that control important cellular signaling pathways such as TGF-β and Wnt signaling, which are inducers of EMT [202]. Another study demonstrated that 10 μM of a mixture of isoflavones (70.5% genistein, 26.3% diadzein, 0.3% glycitein) resulted in the significant upregulation of miRNA 200 and miRNA let-7, as well as significant upregulation of E-cadherin and downregulation of mesenchymal markers, including vemintin, ZEB1 and slug in gemcitabine-resistant pancreatic cancer cells [203]. These results tentatively suggest that polyphenols can reverse EMT and cancer metastasis by altering the expression of miRNAs. Therefore, additional preclinical and clinical studies are needed to focus on the role of polyphenols and their analogs in modulating miRNAs expression and its effect on reversing EMT-mediated metastasis. Such studies would enhance our understanding of the detailed mechanisms of action of polyphenols in cancer metastasis.

4.2. Whole Extract/Combining Polyphenols Studies

Only a few studies have been focused on elucidating the effects of whole polyphenol extracts on EMT, EMT-related pathways and metastasis, while most investigations determined the efficacy of

certain polyphenols isolates or individual compounds. Therefore, future studies should be conducted to determine the efficacy of whole polyphenol extracts in reversing metastasis and identifying the molecular mechanisms. This is important as several studies have reported that the combination of several polyphenolic compounds can further enhance their anticancer efficacy and produce synergistic efficacy against cancer cells proliferation, migration, and invasiveness *in vitro* [204,205]. The combination of polyphenols with other micronutrients, such as vitamin C, was also efficacious [206].

4.3. Use of Doses or Concentrations That Resemble Those Consumed from Dietary Sources

Similar to other natural products, polyphenols are present in dietary sources as complexes with other natural products and chemicals. As indicated earlier, polyphenols are typically extensively metabolized, which further limits their bioavailability. The majority of past studies used high concentrations or doses of polyphenols that were significantly higher than what humans consume in their diet or receive in clinical settings. Therefore, future studies should consider the complex nature of polyphenols, as well as their suboptimal pharmacokinetic profile. It is important to use doses or concentrations that more closely resemble those obtained from dietary consumption in both preclinical and clinical studies to further understand potential health benefits of polyphenols in cancer and other diseases.

5. Conclusions

In conclusion, the anticancer efficacy of polyphenols may be due in part to their effects on the EMT pathway. Polyphenols may also produce their efficacy by inducing apoptosis, increasing ROS levels and modulating the immune system and other mechanisms as previously discussed in this paper. Notably, EMT is particularly important in mediating cancer cell migration and invasive properties, and this can explain, in part, the antimetastatic efficacy of many polyphenolic compounds and mixtures. Different types and mixtures of polyphenols with versatile structural and chemical properties significantly upregulate epithelial markers such as E-cadherin, while significantly downregulate mesenchymal proteins such as N-cadherin, vimentin, and fibronectin. Polyphenols also significantly alter the expression levels of EMT-inducing transcriptional factors such as snail and twist. Importantly, other signaling pathways that interact with the EMT pathway were also affected, possibly explaining the so-called EMT efficacy of the polyphenols. Although efficacious in limiting the EMT process, dietary polyphenols also have limitations that should be considered, including poor and complex biodistribution, kinetics, and metabolic fate *in vivo*. Polyphenols may have limited oral absorption, undergo extensive metabolic degradation, and significantly interact with other ingested drugs and foods, among others. Many of the studies on the effects of polyphenols on EMT signaling are *in vitro* studies that do not reflect the concentrations achieved *in vivo*. Accordingly, additional *in vivo* studies are needed that focus on using concentrations and doses of polyphenols that reflect those consumed from dietary sources and clinical studies which are safe and equivalent to the achieved *in vivo* levels. The identification of specific cellular targets is another limitation. The specific receptors, proteins or transcriptional factors that are directly affected by polyphenols remain to be elucidated.

Acknowledgments: This review was funded by the University of Toledo start-up (F110760) to A.K.T. We would like to thank Charisse Montgomery; University of Toledo for editorial assistance.

Author Contributions: H.A. and A.K.T. proposed the idea and wrote the manuscript; C.R.A., R.P. and T.S. provided critical inputs, edited and proofed the manuscript; A.K.T. supervised the project.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [CrossRef] [PubMed]
2. Lu, J.N.; Lee, W.S.; Kim, M.J.; Yun, J.W.; Jung, J.H.; Yi, S.M.; Jeong, J.-H.; Kim, H.J.; Choi, Y.H.; Kim, G.S. The inhibitory effect of anthocyanins on Akt on invasion and epithelial-mesenchymal transition is not associated with the anti-EGFR effect of the anthocyanins. *Int. J. Oncol.* **2014**, *44*, 1756–1766. [CrossRef] [PubMed]
3. Kundu, J.K.; Chun, K.-S. The promise of dried fruits in cancer chemoprevention. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 3343–3352. [CrossRef] [PubMed]
4. Perez-Jimenez, J.; Neveu, V.; Vos, F.; Scalbert, A. Identification of the 100 richest dietary sources of polyphenols: An application of the phenol-explorer database. *Eur. J. Clin. Nutr.* **2010**, *64*, S112–S120. [CrossRef] [PubMed]
5. Duthie, G.G.; Duthie, S.J.; Kyle, J.A. Plant polyphenols in cancer and heart disease: Implications as nutritional antioxidants. *Nutr. Res. Rev.* **2000**, *13*, 79–106. [CrossRef] [PubMed]
6. Grosso, G.; Godos, J.; Lamuela-Raventos, R.; Ray, S.; Micek, A.; Pajak, A.; Sciacca, S.; D'Orazio, N.; Del Rio, D.; Galvano, F. A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. *Mol. Nutr. Food Res.* **2017**, *61*, 1600930-n/a. [CrossRef] [PubMed]
7. Illam, S.P.; Narayanankutty, A.; Mathew, S.E.; Valsalakumari, R.; Jacob, R.M.; Raghavamenon, A.C. Epithelial Mesenchymal Transition in Cancer Progression: Preventive Phytochemicals. *Recent Pat. Anti-Cancer Drug Discov.* **2017**. [CrossRef]
8. Kim, E.K.; Choi, E.J.; Debnath, T. Role of phytochemicals in the inhibition of epithelial-mesenchymal transition in cancer metastasis. *Food Funct.* **2016**, *7*, 3677–3685. [CrossRef] [PubMed]
9. Kalluri, R.; Weinberg, R.A. The basics of epithelial-mesenchymal transition. *J. Clin. Investig.* **2009**, *119*, 1420–1428. [CrossRef] [PubMed]
10. Thiery, J.P. Epithelial–mesenchymal transitions in tumour progression. *Nat. Rev. Cancer* **2002**, *2*, 442–454. [CrossRef] [PubMed]
11. Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S. [PubMed]
12. Scalbert, A.; Johnson, I.T.; Saltmarsh, M. Polyphenols: Antioxidants and beyond. *Am. J. Clin. Nutr.* **2005**, *81*, 215S–217S. [PubMed]
13. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747. [PubMed]
14. Kahkonen, M.P.; Hopia, A.I.; Vuorela, H.J.; Rauha, J.P.; Pihlaja, K.; Kujala, T.S.; Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **1999**, *47*, 3954–3962. [CrossRef] [PubMed]
15. Harborne, J.B.; Williams, C.A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504. [CrossRef]
16. El Gharras, H. Polyphenols: Food sources, properties and applications—A review. *Int. J. Food Sci. Technol.* **2009**, *44*, 2512–2518. [CrossRef]
17. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*, 1231–1246. [CrossRef] [PubMed]
18. Barbieri, R.; Coppo, E.; Marchese, A.; Daglia, M.; Sobarzo-Sánchez, E.; Nabavi, S.F.; Nabavi, S.M. Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. *Microbiol. Res.* **2017**, *196*, 44–68. [CrossRef] [PubMed]
19. Skalicka-Wozniak, K.; Orhan, I.E.; Cordell, G.A.; Nabavi, S.M.; Budzynska, B. Implication of coumarins towards central nervous system disorders. *Pharmacol. Res.* **2016**, *103*, 188–203. [CrossRef] [PubMed]
20. Menezes, R.; Rodriguez-Mateos, A.; Kaltsatou, A.; González-Sarrías, A.; Greyling, A.; Giannaki, C.; Andres-Lacueva, C.; Milenkovic, D.; Gibney, E.R.; Dumont, J.; et al. Impact of flavonols on cardiometabolic biomarkers: A meta-analysis of randomized controlled human trials to explore the role of inter-individual variability. *Nutrients* **2017**, *9*, 117. [CrossRef] [PubMed]
21. Hostetler, G.L.; Ralston, R.A.; Schwartz, S.J. Flavones: Food sources, bioavailability, metabolism, and bioactivity. *Advances in nutrition (Bethesda, Md.)* **2017**, *8*, 423–435. [CrossRef] [PubMed]

22. Vandeputte, O.M.; Kiendrebeogo, M.; Rasamiravaka, T.; Stevigny, C.; Duez, P.; Rajaonson, S.; Diallo, B.; Mol, A.; Baucher, M.; El Jaziri, M. The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in pseudomonas aeruginosa Pao1. *Microbiology* **2011**, *157*, 2120–2132. [CrossRef] [PubMed]
23. Adlercreutz, H.; Mazur, W. Phyto-oestrogens and western diseases. *Ann. Med.* **1997**, *29*, 95–120. [CrossRef] [PubMed]
24. Ferrazzano, G.F.; Amato, I.; Ingenito, A.; Zarrelli, A.; Pinto, G.; Pollio, A. Plant polyphenols and their anti-cariogenic properties: A review. *Molecules* **2011**, *16*, 1486–1507. [CrossRef] [PubMed]
25. Hara, Y.; Luo, S.; Wickremasinghe, R.; Yamanishi, T. Special issue on tea. *Food Rev. Int.* **1995**, *11*, 371–542.
26. Prior, R.L.; Wu, X. Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic. Res.* **2006**, *40*, 1014–1028. [CrossRef] [PubMed]
27. Wang, Y.; Ho, C.T. Polyphenolic chemistry of tea and coffee: A century of progress. *J. Agric. Food Chem.* **2009**, *57*, 8109–8114. [CrossRef] [PubMed]
28. Clifford, M.N. Chlorogenic acids and other cinnamates—Nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362–372. [CrossRef]
29. Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.W.; Fong, H.H.S.; Farnsworth, N.R.; Kinghorn, A.D.; Mehta, R.G.; et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218–220. [CrossRef] [PubMed]
30. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S. [PubMed]
31. Sies, H.; Schewe, T.; Heiss, C.; Kelm, M. Cocoa polyphenols and inflammatory mediators. *Am. J. Clin. Nutr.* **2005**, *81*, 304S–312S. [PubMed]
32. Vita, J.A. Polyphenols and cardiovascular disease: Effects on endothelial and platelet function. *Am. J. Clin. Nutr.* **2005**, *81*, 292S–297S. [PubMed]
33. Cho, Y.; Schiller, N.; Kahng, H.; Oh, K. Cellular responses and proteomic analysis of *Escherichia coli* exposed to green tea polyphenols. *Curr. Microbiol.* **2007**, *55*, 501–506. [CrossRef] [PubMed]
34. Hubert, P.A.; Lee, S.G.; Lee, S.-K.; Chun, O.K. Dietary polyphenols, berries, and age-related bone loss: A review based on human, animal, and cell studies. *Antioxidants* **2014**, *3*, 144–158. [CrossRef] [PubMed]
35. Lambert, J.D.; Hong, J.; Yang, G.Y.; Liao, J.; Yang, C.S. Inhibition of carcinogenesis by polyphenols: Evidence from laboratory investigations. *Am. J. Clin. Nutr.* **2005**, *81*, 284S–291S. [PubMed]
36. Who, J.; Consultation, F.E. Diet, nutrition and the prevention of chronic diseases. *World Health Organ. Tech. Rep. Ser.* **2003**, *916*, 1–149.
37. Rice-Evans, C. Flavonoid antioxidants. *Curr. Med. Chem.* **2001**, *8*, 797–807. [CrossRef] [PubMed]
38. Upadhyay, S.; Dixit, M. Role of polyphenols and other phytochemicals on molecular signaling. *Oxid. Med. Cell. Longev.* **2015**, *2015*, 504253. [CrossRef] [PubMed]
39. Rechner, A.R.; Kuhnle, G.; Bremner, P.; Hubbard, G.P.; Moore, K.P.; Rice-Evans, C.A. The metabolic fate of dietary polyphenols in humans. *Free Radic. Biol. Med.* **2002**, *33*, 220–235. [CrossRef]
40. Manach, C.; Morand, C.; Crespy, V.; Demigné, C.; Texier, O.; Régérat, F.; Rémésy, C. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett.* **1998**, *426*, 331–336. [CrossRef]
41. Farber, E. The multistep nature of cancer development. *Cancer Res.* **1984**, *44*, 4217–4223. [PubMed]
42. Ramos, S. Cancer chemoprevention and chemotherapy: Dietary polyphenols and signaling pathways. *Mol. Nutr. Food Res.* **2008**, *52*, 507–526. [CrossRef] [PubMed]
43. Fresco, P.; Borges, F.; Marques, M.P.; Diniz, C. The anticancer properties of dietary polyphenols and its relation with apoptosis. *Curr. Pharm. Des.* **2010**, *16*, 114–134. [CrossRef] [PubMed]
44. Amararathna, M.; Johnston, M.R.; Rupasinghe, H.P. Plant polyphenols as chemopreventive agents for lung cancer. *Int. J. Mol. Sci.* **2016**, *17*, 1352. [CrossRef] [PubMed]
45. American Cancer Society. Cancer Facts & Figures 2017. In *Nutrition and Physical Activities*; American Cancer Society: Atlanta, GA, USA, 2017.
46. Turrini, E.; Ferruzzi, L.; Fimognari, C. Potential effects of pomegranate polyphenols in cancer prevention and therapy. *Oxid. Med. Cell. Longev.* **2015**, *2015*, 938475. [CrossRef] [PubMed]
47. Wenzel, U.; Kuntz, S.; Brendel, M.D.; Daniel, H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer Res.* **2000**, *60*, 3823–3831. [PubMed]

48. Yang, C.S.; Landau, J.M.; Huang, M.T.; Newmark, H.L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Ann. Rev. Nutr.* **2001**, *21*, 381–406. [CrossRef] [PubMed]
49. Hadi, S.; Asad, S.; Singh, S.; Ahmad, A. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. *IUBMB Life Sci.* **2000**, *50*, 167–171.
50. Wessner, B.; Strasser, E.M.; Koitz, N.; Schmuckenschlager, C.; Unger-Manhart, N.; Roth, E. Green tea polyphenol administration partly ameliorates chemotherapy-induced side effects in the small intestine of mice. *J. Nutr.* **2007**, *137*, 634–640. [PubMed]
51. Harper, C.E.; Patel, B.B.; Wang, J.; Eltoum, I.A.; Lamartiniere, C.A. Epigallocatechin-3-gallate suppresses early stage, but not late stage prostate cancer in tramp mice: Mechanisms of action. *Prostate* **2007**, *67*, 1576–1589. [CrossRef] [PubMed]
52. Chuang, S.E.; Cheng, A.L.; Lin, J.K.; Kuo, M.L. Inhibition by curcumin of diethylnitrosamine-induced hepatic hyperplasia, inflammation, cellular gene products and cell-cycle-related proteins in rats. *Food Chem. Toxicol.* **2000**, *38*, 991–995. [CrossRef]
53. Surh, Y.J. Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* **2003**, *3*, 768–780. [CrossRef] [PubMed]
54. Weng, C.-J.; Yen, G.-C. Chemopreventive effects of dietary phytochemicals against cancer invasion and metastasis: Phenolic acids, monophenol, polyphenol, and their derivatives. *Cancer Treat. Rev.* **2012**, *38*, 76–87. [CrossRef] [PubMed]
55. Chen, D.; Daniel, K.G.; Kuhn, D.J.; Kazi, A.; Bhuiyan, M.; Li, L.; Wang, Z.; Wan, S.B.; Lam, W.H.; Chan, T.H. Green tea and tea polyphenols in cancer prevention. *Front Biosci.* **2004**, *9*, 2618–2631. [CrossRef] [PubMed]
56. Link, A.; Balaguer, F.; Goel, A. Cancer chemoprevention by dietary polyphenols: Promising role for epigenetics. *Biochem. Pharmacol.* **2010**, *80*, 1771–1792. [CrossRef] [PubMed]
57. Tsuji, P.A.; Walle, T. Inhibition of benzo [a] pyrene-activating enzymes and DNA binding in human bronchial epithelial BEAS-2B cells by methoxylated flavonoids. *Carcinogenesis* **2006**, *27*, 1579–1585. [CrossRef] [PubMed]
58. Zhai, X.; Lin, M.; Zhang, F.; Hu, Y.; Xu, X.; Li, Y.; Liu, K.; Ma, X.; Tian, X.; Yao, J. Dietary flavonoid genistein induces NRF2 and Phase II detoxification gene expression via ERKS and PKC pathways and protects against oxidative stress in CACO-2 cells. *Mol. Nutr. Food Res.* **2013**, *57*, 249–259. [CrossRef] [PubMed]
59. Murakami, C.; Hirakawa, Y.; Nakano, Y.; Yoshida, H. Effects of epigallocatechin 3-O-gallate on cellular antioxidative system in HepG2 cells. *J. Nutr. Sci. Vitaminol.* **2002**, *48*, 89–94. [CrossRef] [PubMed]
60. Schwarz, D.; Roots, I. In vitro assessment of inhibition by natural polyphenols of metabolic activation of procarcinogens by human CYP1A1. *Biochem. Biophys. Res. Commun.* **2003**, *303*, 902–907. [CrossRef]
61. Howells, L.M.; Mitra, A.; Manson, M.M. Comparison of oxaliplatin-and curcumin-mediated antiproliferative effects in colorectal cell lines. *Int. J. Cancer* **2007**, *121*, 175–183. [CrossRef] [PubMed]
62. Nakazato, T.; Ito, K.; Ikeda, Y.; Kizaki, M. Green tea component, catechin, induces apoptosis of human malignant B cells via production of reactive oxygen species. *Clin. Cancer Res.* **2005**, *11*, 6040–6049. [CrossRef] [PubMed]
63. Balasubramanian, S.; Efimova, T.; Eckert, R.L. Green tea polyphenol stimulates a Ras, Mekk1, Mek3, and p38 cascade to increase activator protein 1 factor-dependent involucrin gene expression in normal human keratinocytes. *J. Biol. Chem.* **2002**, *277*, 1828–1836. [CrossRef] [PubMed]
64. Gong, L.; Li, Y.; Nedeljkovic-Kurepa, A.; Sarkar, F.H. Inactivation of NF-κB by genistein is mediated via Akt signaling pathway in breast cancer cells. *Oncogene* **2003**, *22*, 4702–4709. [CrossRef] [PubMed]
65. Ye, F.; Wu, J.; Dunn, T.; Yi, J.; Tong, X.; Zhang, D. Inhibition of cyclooxygenase-2 activity in head and neck cancer cells by genistein. *Cancer Lett.* **2004**, *211*, 39–46. [CrossRef] [PubMed]
66. Adams, L.S.; Seeram, N.P.; Aggarwal, B.B.; Takada, Y.; Sand, D.; Heber, D. Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. *J. Agric. Food Chem.* **2006**, *54*, 980–985. [CrossRef] [PubMed]
67. Kao, Y.-L.; Kuo, Y.-M.; Lee, Y.-R.; Yang, S.-F.; Chen, W.-R.; Lee, H.-J. Apple polyphenol induces cell apoptosis, cell cycle arrest at G2/m phase, and mitotic catastrophe in human bladder transitional carcinoma cells. *J. Funct. Foods* **2015**, *14*, 384–394. [CrossRef]
68. Singh, M.; Singh, R.; Bhui, K.; Tyagi, S.; Mahmood, Z.; Shukla, Y. Tea polyphenols induce apoptosis through mitochondrial pathway and by inhibiting nuclear factor-kappa B and Akt activation in human cervical cancer cells. *Oncol. Res.* **2011**, *19*, 245–257. [CrossRef] [PubMed]

69. Monasterio, A.; Urdaci, M.C.; Pinchuk, I.V.; Lopez-Moratalla, N.; Martinez-Irujo, J.J. Flavonoids induce apoptosis in human leukemia u937 cells through caspase-and caspase-calpain-dependent pathways. *Nutr. Cancer* **2004**, *50*, 90–100. [CrossRef] [PubMed]
70. Brusselmans, K.; Vrolix, R.; Verhoeven, G.; Swinnen, J.V. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. *J. Biol. Chem.* **2005**, *280*, 5636–5645. [CrossRef] [PubMed]
71. Lee, S.H.; Yumnam, S.; Hong, G.E.; Raha, S.; Saralamma, V.V.; Lee, H.J.; Heo, J.D.; Lee, S.J.; Lee, W.S.; Kim, E.H.; et al. Flavonoids of korean citrus *aurantium* L. induce apoptosis via intrinsic pathway in human hepatoblastoma HepG2 cells. *Phytother. Res. PTR* **2015**, *29*, 1940–1949. [CrossRef] [PubMed]
72. Miquel, J.; Bernd, A.; Sempere, J.M.; Diaz-Alperi, J.; Ramirez, A. The curcuma antioxidants: Pharmacological effects and prospects for future clinical use. A review. *Arch. Gerontol. Geriatr.* **2002**, *34*, 37–46. [CrossRef]
73. Sarkar, F.H.; Li, Y.; Wang, Z.; Kong, D. The role of nutraceuticals in the regulation of Wnt and hedgehog signaling in cancer. *Cancer Metastasis Rev.* **2010**, *29*, 383–394. [CrossRef] [PubMed]
74. George, V.C.; Dellaire, G.; Rupasinghe, H.P.V. Plant flavonoids in cancer chemoprevention: Role in genome stability. *J. Nutr. Biochem.* **2017**, *45*, 1–14. [CrossRef] [PubMed]
75. Garcia, R.; Gonzalez, C.A.; Agudo, A.; Riboli, E. High intake of specific carotenoids and flavonoids does not reduce the risk of bladder cancer. *Nutr. Cancer* **1999**, *35*, 212–214. [CrossRef] [PubMed]
76. Arts, I.C.; Hollman, P.C.; Bueno De Mesquita, H.B.; Feskens, E.J.; Kromhout, D. Dietary catechins and epithelial cancer incidence: The zutphen elderly study. *Int. J. Cancer* **2001**, *92*, 298–302. [CrossRef]
77. Mehlen, P.; Puisieux, A. Metastasis: A question of life or death. *Nature Reviews Cancer* **2006**, *6*, 449–458. [CrossRef] [PubMed]
78. Kohn, E.C. Development and prevention of metastasis. *Anticancer Res.* **1992**, *13*, 2553–2559.
79. Ali, S.; Lazennec, G. Chemokines: Novel targets for breast cancer metastasis. *Cancer Metastasis Rev.* **2007**, *26*, 401–420. [CrossRef] [PubMed]
80. Nguyen, D.X.; Bos, P.D.; Massague, J. Metastasis: From dissemination to organ-specific colonization. *Nat. Rev. Cancer* **2009**, *9*, 274–284. [CrossRef] [PubMed]
81. Jiang, W.G.; Sanders, A.J.; Katoh, M.; Ungefroren, H.; Gieseler, F.; Prince, M.; Thompson, S.K.; Zollo, M.; Spano, D.; Dhawan, P.; et al. Tissue invasion and metastasis: Molecular, biological and clinical perspectives. *Semin. Cancer Biol.* **2015**, *35*, S244–S275. [CrossRef] [PubMed]
82. Shih, J.-Y.; Yang, S.-C.; Hong, T.-M.; Yuan, A.; Chen, J.J.; Yu, C.-J.; Chang, Y.-L.; Lee, Y.-C.; Peck, K.; Wu, C.-W. Collapsin response mediator protein-1 and the invasion and metastasis of cancer cells. *J. Natl. Cancer Inst.* **2001**, *93*, 1392–1400. [CrossRef] [PubMed]
83. Orr, F.W.; Wang, H.H.; Lafrenie, R.M.; Scherbarth, S.; Nance, D.M. Interactions between cancer cells and the endothelium in metastasis. *J. Pathol.* **2000**, *190*, 310–329. [CrossRef]
84. Clark, E.A.; Golub, T.R.; Lander, E.S.; Hynes, R.O. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **2000**, *406*, 532–535. [CrossRef] [PubMed]
85. Mikami, S.; Mizuno, R.; Kosaka, T.; Saya, H.; Oya, M.; Okada, Y. Expression of TNF-α and CD44 is implicated in poor prognosis, cancer cell invasion, metastasis and resistance to the sunitinib treatment in clear cell renal cell carcinomas. *Int. J. Cancer* **2015**, *136*, 1504–1514. [CrossRef] [PubMed]
86. Deryugina, E.I.; Quigley, J.P. Tumor angiogenesis: Mmp-mediated induction of intravasation- and metastasis-sustaining neovasculature. *Matrix Biol.* **2015**, *44–46*, 94–112. [CrossRef] [PubMed]
87. Slaney, C.Y.; Rautela, J.; Parker, B.S. The emerging role of immunosurveillance in dictating metastatic spread in breast cancer. *Cancer Res.* **2013**, *73*, 5852–5857. [CrossRef] [PubMed]
88. Gordon, M.A.; Zhang, W.; Yang, D.; Iqbal, S.; El-Khouiery, A.; Nagashima, F.; Lurje, G.; Labonte, M.; Wilson, P.; Sherrod, A.; et al. Gender-specific genomic profiling in metastatic colorectal cancer patients treated with 5-fluorouracil and oxaliplatin. *Pharmacogenomics* **2011**, *12*, 27–39. [CrossRef] [PubMed]
89. Nguyen, D.X.; Massague, J. Genetic determinants of cancer metastasis. *Nat. Rev. Genet.* **2007**, *8*, 341–352. [CrossRef] [PubMed]
90. National Cancer Institute. *Metastatic Cancer*; National institute of cancer at the National institute of Health: Bethesda, MD, USA, 2017.
91. Liang, X. EMT: New signals from the invasive front. *Oral Oncol.* **2011**, *47*, 686–687. [CrossRef] [PubMed]
92. Polyak, K.; Weinberg, R.A. Transitions between epithelial and mesenchymal states: Acquisition of malignant and stem cell traits. *Nat. Rev. Cancer* **2009**, *9*, 265–273. [CrossRef] [PubMed]

93. Lamouille, S.; Xu, J.; Derynck, R. Molecular mechanisms of epithelial—Mesenchymal transition. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 178–196. [CrossRef] [PubMed]
94. Onder, T.T.; Gupta, P.B.; Mani, S.A.; Yang, J.; Lander, E.S.; Weinberg, R.A. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res.* **2008**, *68*, 3645–3654. [CrossRef] [PubMed]
95. Nelson, W.J.; Nusse, R. Convergence of Wnt, ß-catenin, and cadherin pathways. *Science* **2004**, *303*, 1483–1487. [CrossRef] [PubMed]
96. Xie, L.; Law, B.K.; Chytil, A.M.; Brown, K.A.; Aakre, M.E.; Moses, H.L. Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. *Neoplasia* **2004**, *6*, 603–610. [CrossRef] [PubMed]
97. Boyer, B.; Vallés, A.M.; Edme, N. Induction and regulation of epithelial—Mesenchymal transitions. *Biochem. Pharmacol.* **2000**, *60*, 1091–1099. [CrossRef]
98. Willis, B.C.; Borok, Z. TGF-β-induced EMT: Mechanisms and implications for fibrotic lung disease. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **2007**, *293*, L525–L534. [CrossRef] [PubMed]
99. Graham, T.R.; Zhau, H.E.; Odero-Marah, V.A.; Osunkoya, A.O.; Kimbro, K.S.; Tighiouart, M.; Liu, T.; Simons, J.W.; O'Regan, R.M. Insulin-like growth factor-I–dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res.* **2008**, *68*, 2479–2488. [CrossRef] [PubMed]
100. Lo, H.-W.; Hsu, S.-C.; Xia, W.; Cao, X.; Shih, J.-Y.; Wei, Y.; Abbruzzese, J.L.; Hortobagyi, G.N.; Hung, M.-C. Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of Twist gene expression. *Cancer Res.* **2007**, *67*, 9066–9076. [CrossRef] [PubMed]
101. Kong, D.; Wang, Z.; Sarkar, S.H.; Li, Y.; Banerjee, S.; Saliganan, A.; Kim, H.R.C.; Cher, M.L.; Sarkar, F.H. Platelet-derived growth factor-d overexpression contributes to epithelial-mesenchymal transition of PC3 prostate cancer cells. *Stem Cells* **2008**, *26*, 1425–1435. [CrossRef] [PubMed]
102. Julien, S.; Puig, I.; Caretti, E.; Bonaventure, J.; Nelles, L.; Van Roy, F.; Dargemont, C.; De Herreros, A.G.; Bellacosa, A.; Larue, L. Activation of NF-κb by Akt upregulates snail expression and induces epithelium mesenchyme transition. *Oncogene* **2007**, *26*, 7445–7456. [CrossRef] [PubMed]
103. Grille, S.J.; Bellacosa, A.; Upson, J.; Klein-Szanto, A.J.; Van Roy, F.; Lee-Kwon, W.; Donowitz, M.; Tsichlis, P.N.; Larue, L. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. *Cancer Res.* **2003**, *63*, 2172–2178. [PubMed]
104. Kim, K.; Lu, Z.; Hay, E.D. Direct evidence for a role of β-catenin/lef-1 signaling pathway in induction of EMT. *Cell Biol. Int.* **2002**, *26*, 463–476. [CrossRef] [PubMed]
105. Hu, C.-T.; Wu, J.-R.; Chang, T.Y.; Cheng, C.-C.; Wu, W.-S. The transcriptional factor snail simultaneously triggers cell cycle arrest and migration of human hepatoma HepG2. *J. Biomed. Sci.* **2008**, *15*, 343–355. [CrossRef] [PubMed]
106. Peinado, H.; Marin, F.; Cubillo, E.; Stark, H.-J.; Fusenig, N.; Nieto, M.A.; Cano, A. Snail and E47 repressors of E-cadherin induce distinct invasive and angiogenic properties in vivo. *J. Cell Sci.* **2004**, *117*, 2827–2839. [CrossRef] [PubMed]
107. Spaderna, S.; Schmalhofer, O.; Wahlbuhl, M.; Dimmler, A.; Bauer, K.; Sultan, A.; Hlubek, F.; Jung, A.; Strand, D.; Eger, A. The transcriptional repressor Zeb1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res.* **2008**, *68*, 537–544. [CrossRef] [PubMed]
108. Yang, J.; Mani, S.A.; Donaher, J.L.; Ramaswamy, S.; Itzykson, R.A.; Come, C.; Savagner, P.; Gitelman, I.; Richardson, A.; Weinberg, R.A. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **2004**, *117*, 927–939. [CrossRef] [PubMed]
109. Yao, D.; Dai, C.; Peng, S. Mechanism of the mesenchymal–epithelial transition and its relationship with metastatic tumor formation. *Mol. Cancer Res.* **2011**, *9*, 1608–1620. [CrossRef] [PubMed]
110. Brembeck, F.H.; Rosário, M.; Birchmeier, W. Balancing cell adhesion and wnt signaling, the key role of β-catenin. *Curr. Opin. Genet. Dev.* **2006**, *16*, 51–59. [CrossRef] [PubMed]
111. MacDonald, B.T.; Tamai, K.; He, X. Wnt/β-catenin signaling: Components, mechanisms, and diseases. *Dev. Cell* **2009**, *17*, 9–26. [CrossRef] [PubMed]
112. Clevers, H. Wnt/β-catenin signaling in development and disease. *Cell* **2006**, *127*, 469–480. [CrossRef] [PubMed]
113. Eastman, Q.; Grosschedl, R. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Curr. Opin. Cell Biol.* **1999**, *11*, 233–240. [CrossRef]

114. Roose, J.; Huls, G.; Van Beest, M.; Moerer, P.; Van Der Horn, K.; Goldschmeding, R.; Logtenberg, T.; Clevers, H. Synergy between tumor suppressor APC and the β-catenin-TCF4 target TCF1. *Science* **1999**, *285*, 1923–1926. [CrossRef] [PubMed]
115. Van De Wetering, M.; Sancho, E.; Verweij, C.; De Lau, W.; Oving, I.; Hurlstone, A.; Van Der Horn, K.; Batlle, E.; Coudreuse, D.; Haramis, A.-P. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **2002**, *111*, 241–250. [CrossRef]
116. Davidson, G.; Wu, W.; Shen, J.; Bilic, J.; Fenger, U.; Stannek, P.; Glinka, A.; Niehrs, C. Casein kinase 1 γ couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* **2005**, *438*, 867–872. [CrossRef] [PubMed]
117. Orsulic, S.; Huber, O.; Aberle, H.; Arnold, S.; Kemler, R. E-cadherin binding prevents beta-catenin nuclear localization and beta-catenin/LEF-1-mediated transactivation. *J. Cell Sci.* **1999**, *112*, 1237–1245. [PubMed]
118. Chang, Y.-W.; Su, Y.-J.; Hsiao, M.; Wei, K.-C.; Lin, W.-H.; Liang, C.-J.; Chen, S.-C.; Lee, J.-L. Diverse targets of β-catenin during the epithelial–mesenchymal transition define cancer stem cells and predict disease relapse. *Cancer Res.* **2015**, *75*, 3398–3410. [CrossRef] [PubMed]
119. Ozawa, M.; Baribault, H.; Kemler, R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* **1989**, *8*, 1711. [PubMed]
120. Cano, A.; Pérez-Moreno, M.A.; Rodrigo, I.; Locascio, A.; Blanco, M.J.; del Barrio, M.G.; Portillo, F.; Nieto, M.A. The transcription factor snail controls epithelial—Mesenchymal transitions by repressing E-cadherin expression. *Nat. Cell Biol.* **2000**, *2*, 76–83. [CrossRef] [PubMed]
121. Stemmer, V.; De Craene, B.; Berx, G.; Behrens, J. Snail promotes Wnt target gene expression and interacts with β-catenin. *Oncogene* **2008**, *27*, 5075–5080. [CrossRef] [PubMed]
122. Yook, J.I.; Li, X.-Y.; Ota, I.; Fearon, E.R.; Weiss, S.J. Wnt-dependent regulation of the E-cadherin repressor snail. *J. Biol. Chem.* **2005**, *280*, 11740–11748. [CrossRef] [PubMed]
123. Zhou, B.P.; Deng, J.; Xia, W.; Xu, J.; Li, Y.M.; Gunduz, M.; Hung, M.-C. Dual regulation of snail by GSK-3β-mediated phosphorylation in control of epithelial–mesenchymal transition. *Nat. Cell Biol.* **2004**, *6*, 931–940. [CrossRef] [PubMed]
124. Zucchini-Pascal, N.; Peyre, L.; Rahmani, R. Crosstalk between beta-catenin and snail in the induction of epithelial to mesenchymal transition in hepatocarcinoma: Role of the Erk1/2 pathway. *Int. J. Mol. Sci.* **2013**, *14*, 20768–20792. [CrossRef] [PubMed]
125. Huang, J.; Li, H.; Ren, G. Epithelial-mesenchymal transition and drug resistance in breast cancer (review). *Int. J. Oncol.* **2015**, *47*, 840–848. [CrossRef] [PubMed]
126. Sommers, C.L.; Heckford, S.E.; Skerker, J.M.; Worland, P.; Torri, J.A.; Thompson, E.W.; Byers, S.W.; Gelmann, E.P. Loss of epithelial markers and acquisition of vimentin expression in adriamycin-and vinblastine-resistant human breast cancer cell lines. *Cancer Res.* **1992**, *52*, 5190–5197. [PubMed]
127. Fischer, K.R.; Durrans, A.; Lee, S.; Sheng, J.; Li, F.; Wong, S.T.; Choi, H.; El Rayes, T.; Ryu, S.; Troeger, J. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* **2015**, *527*, 472–476. [CrossRef] [PubMed]
128. Du, B.; Shim, J.S. Targeting epithelial–mesenchymal transition (EMT) to overcome drug resistance in cancer. *Molecules* **2016**, *21*, 965. [CrossRef] [PubMed]
129. Kampa, M.; Nifli, A.P.; Notas, G.; Castanas, E. Polyphenols and cancer cell growth. *Rev. Physiol. Biochem. Pharmacol.* **2007**, *159*, 79–113. [PubMed]
130. Zhou, Q.; Bennett, L.L.; Zhou, S. Multifaceted ability of naturally occurring polyphenols against metastatic cancer. *Clin. Exp. Pharmacol. Physiol.* **2016**, *43*, 394–409. [CrossRef] [PubMed]
131. Kita, Y.; Miura, Y.; Yagasaki, K. Antiproliferative and anti-invasive effect of piceatannol, a polyphenol present in grapes and wine, against hepatoma AH109A cells. *BioMed Res. Int.* **2012**, *2012*, 672416. [CrossRef] [PubMed]
132. Lee, S.H.; Jaganath, I.B.; Wang, S.M.; Sekaran, S.D. Antimetastatic effects of phyllanthus on human lung (A549) and breast (MCF-7) cancer cell lines. *PLoS ONE* **2011**, *6*, e20994. [CrossRef] [PubMed]
133. Vergara, D.; Simeone, P.; Bettini, S.; Tinelli, A.; Valli, L.; Storelli, C.; Leo, S.; Santino, A.; Maffia, M. Antitumor activity of the dietary diterpene carnosol against a panel of human cancer cell lines. *Food Funct.* **2014**, *5*, 1261–1269. [CrossRef] [PubMed]
134. Lee, S.J.; Chung, I.M.; Kim, M.Y.; Park, K.D.; Park, W.W.; Moon, H.I. Inhibition of lung metastasis in mice by oligonol. *Phytother. Res.* **2009**, *23*, 1043–1046. [CrossRef] [PubMed]

135. Ho, Y.C.; Yang, S.F.; Peng, C.Y.; Chou, M.Y.; Chang, Y.C. Epigallocatechin-3-gallate inhibits the invasion of human oral cancer cells and decreases the productions of matrix metalloproteinases and urokinase-plasminogen activator. *J. Oral Pathol. Med.* **2007**, *36*, 588–593. [CrossRef] [PubMed]
136. Sun, C.-Y.; Hu, Y.; Guo, T.; Wang, H.-F.; Zhang, X.-P.; He, W.-J.; Tan, H. Resveratrol as a novel agent for treatment of multiple myeloma with matrix metalloproteinase inhibitory activity. *Acta Pharmacol. Sin.* **2006**, *27*, 1447. [CrossRef] [PubMed]
137. Bigelow, R.L.H.; Cardelli, J.A. The green tea catechins, (−)-epigallocatechin-3-gallate (EGCG) and (−)-epicatechin-3-gallate (ECG), inhibit HGF//Met signaling in immortalized and tumorigenic breast epithelial cells. *Oncogene* **2006**, *25*, 1922–1930. [CrossRef] [PubMed]
138. Milligan, S.A.; Burke, P.; Coleman, D.T.; Bigelow, R.L.; Steffan, J.J.; Carroll, J.L.; Williams, B.J.; Cardelli, J.A. The green tea polyphenol EGCG potentiates the antiproliferative activity of c-met and epidermal growth factor receptor inhibitors in non–small cell lung cancer cells. *Clin. Cancer Res.* **2009**, *15*, 4885–4894. [CrossRef] [PubMed]
139. Bachmeier, B.E.; Nerlich, A.G.; Iancu, C.M.; Cilli, M.; Schleicher, E.; Vené, R.; Dell'Eva, R.; Jochum, M.; Albini, A.; Pfeffer, U. The chemopreventive polyphenol curcumin prevents hematogenous breast cancer metastases in immunodeficient mice. *Cell. Physiol. Biochem.* **2007**, *19*, 137–152. [CrossRef] [PubMed]
140. Sung, B.; Pandey, M.K.; Nakajima, Y.; Nishida, H.; Konishi, T.; Chaturvedi, M.M.; Aggarwal, B.B. Identification of a novel blocker of Iκbα kinase activation that enhances apoptosis and inhibits proliferation and invasion by suppressing nuclear factor-κb. *Mol. Cancer Ther.* **2008**, *7*, 191–201. [CrossRef] [PubMed]
141. Zetter, P.; Bruce, R. Angiogenesis and tumor metastasis. *Ann. Rev. Med.* **1998**, *49*, 407–424. [CrossRef] [PubMed]
142. Terzuoli, E.; Donnini, S.; Giachetti, A.; Iñiguez, M.A.; Fresno, M.; Melillo, G.; Ziche, M. Inhibition of hypoxia inducible factor-1α by dihydroxyphenylethanol, a product from olive oil, blocks microsomal prostaglandin-E synthase-1/vascular endothelial growth factor expression and reduces tumor angiogenesis. *Clin. Cancer Res.* **2010**, *16*, 4207–4216. [CrossRef] [PubMed]
143. Ko, Y.S.; Lee, W.S.; Panchanathan, R.; Joo, Y.N.; Choi, Y.H.; Kim, G.S.; Jung, J.M.; Ryu, C.H.; Shin, S.C.; Kim, H.J. Polyphenols from *Artemisia annua* L. inhibit adhesion and EMT of highly metastatic breast cancer cells MDA-MB-231. *Phytother. Res.* **2016**, *30*, 1180–1188. [CrossRef] [PubMed]
144. Ko, Y.S.; Lee, W.S.; Joo, Y.N.; Choi, Y.H.; Kim, G.S.; Jung, J.-M.; Ryu, C.H.; Shin, S.C.; Kim, H.J. Polyphenol mixtures of euphorbia supina the inhibit invasion and metastasis of highly metastatic breast cancer MDA-MB-231 cells. *Oncol. Rep.* **2015**, *34*, 3035–3042. [CrossRef] [PubMed]
145. Zhang, Z.; Chen, H.; Xu, C.; Song, L.; Huang, L.; Lai, Y.; Wang, Y.; Chen, H.; Gu, D.; Ren, L. Curcumin inhibits tumor epithelial-mesenchymal transition by downregulating the Wnt signaling pathway and upregulating NKD2 expression in colon cancer cells. *Oncol. Rep.* **2016**, *35*, 2615–2623. [CrossRef] [PubMed]
146. Amawi, H.; Ashby, C.R.; Tiwari, A.K. Cancer chemoprevention through dietary flavonoids: What's limiting? *Chin. J. Cancer* **2017**, *36*, 50. [CrossRef] [PubMed]
147. Hertog, M.G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Int. Med.* **1995**, *155*, 381–386. [CrossRef]
148. Nijveldt, R.J.; Van Nood, E.; Van Hoorn, D.E.; Boelens, P.G.; Van Norren, K.; Van Leeuwen, P.A. Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425. [PubMed]
149. Kang, J.; Kim, E.; Kim, W.; Seong, K.M.; Youn, H.; Kim, J.W.; Kim, J.; Youn, B. Rhamnetin and cirsiliol induce radiosensitization and inhibition of epithelial-mesenchymal transition (EMT) by MIR-34A-mediated suppression of notch-1 expression in non-small cell lung cancer cell lines. *J. Biol. Chem.* **2013**, *288*, 27343–27357. [CrossRef] [PubMed]
150. Lin, C.-H.; Shen, Y.-A.; Hung, P.-H.; Yu, Y.-B.; Chen, Y.-J. Epigallocathechin gallate, polyphenol present in green tea, inhibits stem-like characteristics and epithelial-mesenchymal transition in nasopharyngeal cancer cell lines. *BMC Complement. Altern. Med.* **2012**, *12*, 201. [CrossRef] [PubMed]
151. Lin, Y.S.; Tsai, P.H.; Kandaswami, C.C.; Cheng, C.H.; Ke, F.C.; Lee, P.P.; Hwang, J.J.; Lee, M.T. Effects of dietary flavonoids, luteolin, and quercetin on the reversal of epithelial-mesenchymal transition in A431 epidermal cancer cells. *Cancer Sci.* **2011**, *102*, 1829–1839. [CrossRef] [PubMed]
152. Chong, J.; Poutaraud, A.; Hugueney, P. Metabolism and roles of stilbenes in plants. *Plant Sci.* **2009**, *177*, 143–155. [CrossRef]

153. Ji, Q.; Liu, X.; Han, Z.; Zhou, L.; Sui, H.; Yan, L.; Jiang, H.; Ren, J.; Cai, J.; Li, Q. Resveratrol suppresses epithelial-to-mesenchymal transition in colorectal cancer through TGF-β1/Smads signaling pathway mediated snail/E-cadherin expression. *BMC Cancer* **2015**, *15*, 97. [CrossRef] [PubMed]
154. Vergara, D.; Valente, C.M.; Tinelli, A.; Siciliano, C.; Lorusso, V.; Acierno, R.; Giovinazzo, G.; Santino, A.; Storelli, C.; Maffia, M. Resveratrol inhibits the epidermal growth factor-induced epithelial mesenchymal transition in MCF-7 cells. *Cancer Lett.* **2011**, *310*, 1–8. [CrossRef] [PubMed]
155. Gao, Q.; Yuan, Y.; Gan, H.Z.; Peng, Q. Resveratrol inhibits the hedgehog signaling pathway and epithelial-mesenchymal transition and suppresses gastric cancer invasion and metastasis. *Oncol. Lett.* **2015**, *9*, 2381–2387. [CrossRef] [PubMed]
156. Baribeau, S.; Chaudhry, P.; Parent, S.; Asselin, É. Resveratrol inhibits cisplatin-induced epithelial-to-mesenchymal transition in ovarian cancer cell lines. *PLoS ONE* **2014**, *9*, e86987. [CrossRef] [PubMed]
157. Vergara, D.; De Domenico, S.; Tinelli, A.; Stanca, A.; Del Mercato, L.L.; Giudetti, A.M.; Simeone, P.; Guazzelli, N.; Lessi, M.; Manzini, C.; et al. Anticancer effects of novel resveratrol analogues on human ovarian cancer cells. *Mol. Biosyst.* **2017**, *13*, 1131–1141. [CrossRef] [PubMed]
158. Zhou, Y.; Zheng, J.; Li, Y.; Xu, D.-P.; Li, S.; Chen, Y.-M.; Li, H.-B. Natural polyphenols for prevention and treatment of cancer. *Nutrients* **2016**, *8*, 515. [CrossRef] [PubMed]
159. Lall, R.K.; Syed, D.N.; Adhami, V.M.; Khan, M.I.; Mukhtar, H. Dietary polyphenols in prevention and treatment of prostate cancer. *Int. J. Mol. Sci.* **2015**, *16*, 3350–3376. [CrossRef] [PubMed]
160. Carocho, M.; Ferreira, I.C. The role of phenolic compounds in the fight against cancer—A review. *Anti-Cancer Agents Med. Chem.* **2013**, *13*, 1236–1258. [CrossRef]
161. Anantharaju, P.G.; Gowda, P.C.; Vimalambike, M.G.; Madhunapantula, S.V. An overview on the role of dietary phenolics for the treatment of cancers. *Nutr. J.* **2016**, *15*, 99. [CrossRef] [PubMed]
162. Niero, E.L.; Machado-Santelli, G.M. Cinnamic acid induces apoptotic cell death and cytoskeleton disruption in human melanoma cells. *J. Exp. Clin. Cancer Res.* **2013**, *32*, 31. [CrossRef] [PubMed]
163. Zhao, B.; Hu, M. Gallic acid reduces cell viability, proliferation, invasion and angiogenesis in human cervical cancer cells. *Oncol. Lett.* **2013**, *6*, 1749–1755. [PubMed]
164. Rajendra Prasad, N.; Karthikeyan, A.; Karthikeyan, S.; Reddy, B.V. Inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line. *Mol. Cell. Biochem.* **2011**, *349*, 11–19. [CrossRef] [PubMed]
165. Zhang, X.; Lin, D.; Jiang, R.; Li, H.; Wan, J.; Li, H. Ferulic acid exerts antitumor activity and inhibits metastasis in breast cancer cells by regulating epithelial to mesenchymal transition. *Oncol. Rep.* **2016**, *36*, 271–278. [CrossRef] [PubMed]
166. Wu, Y.; He, L.; Zhang, L.; Chen, J.; Yi, Z.; Zhang, J.; Liu, M.; Pang, X. Anacardic acid (6-pentadecylsalicylic acid) inhibits tumor angiogenesis by targeting Src/Fak/Rho GTPases signaling pathway. *J. Pharmacol. Exp. Ther.* **2011**, *339*, 403–411. [CrossRef] [PubMed]
167. Yao, K.; Jiang, X.; He, L.; Tang, Y.; Yin, G.; Zeng, Q.; Jiang, Z.; Tan, J. Anacardic acid sensitizes prostate cancer cells to radiation therapy by regulating h2ax expression. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 15926–15932. [PubMed]
168. Yang, Y.; Li, Y.; Wang, K.; Wang, Y.; Yin, W.; Li, L. P38/NF-kb-/Snail pathway is involved in caffeic acid-induced inhibition of cancer stem cells-like properties and migratory capacity in malignant human keratinocyte. *PLoS ONE* **2013**, *8*, e58915.
169. Wei, M.G.; Sun, W.; He, W.M.; Ni, L.; Yang, Y.Y. Ferulic acid attenuates TGF-beta1-induced renal cellular fibrosis in NRK-52E cells by inhibiting smad/ILK/snail pathway. *Evid.-Based Complement. Altern. Med.* **2015**, *2015*, 619720. [CrossRef] [PubMed]
170. Faried, A.; Kurnia, D.; Faried, L.S.; Usman, N.; Miyazaki, T.; Kato, H.; Kuwano, H. Anticancer effects of gallic acid isolated from indonesian herbal medicine, *Phaleria macrocarpa* (Scheff.) Boerl, on human cancer cell lines. *Int. J. Oncol.* **2007**, *30*, 605–613. [CrossRef] [PubMed]
171. Su, T.R.; Lin, J.J.; Tsai, C.C.; Huang, T.K.; Yang, Z.Y.; Wu, M.O.; Zheng, Y.Q.; Su, C.C.; Wu, Y.J. Inhibition of melanogenesis by gallic acid: Possible involvement of the pi3k/akt, mek/erk and wnt/beta-catenin signaling pathways in b16f10 cells. *Int. J. of Mol. Sci.* **2013**, *14*, 20443–20458. [CrossRef] [PubMed]
172. Wang, L.; Li, W.; Lin, M.; Garcia, M.; Mulholland, D.; Lilly, M.; Martins-Green, M. Luteolin, ellagic acid and punicic acid are natural products that inhibit prostate cancer metastasis. *Carcinogenesis* **2014**, *35*, 2321–2330. [CrossRef] [PubMed]

173. Lo, C.; Lai, T.Y.; Yang, J.S.; Yang, J.H.; Ma, Y.S.; Weng, S.W.; Lin, H.Y.; Chen, H.Y.; Lin, J.G.; Chung, J.G. Gallic acid inhibits the migration and invasion of A375.S2 human melanoma cells through the inhibition of matrix metalloproteinase-2 and Ras. *Melanoma Res.* **2011**, *21*, 267–273. [CrossRef] [PubMed]
174. Teponno, R.B.; Kusari, S.; Spiteller, M. Recent advances in research on lignans and neolignans. *Nat. prod. Rep.* **2016**, *33*, 1044–1092. [CrossRef] [PubMed]
175. Milder, I.E.; Arts, I.C.; van de Putte, B.; Venema, D.P.; Hollman, P.C. Lignan contents of Dutch plant foods: A database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. *Br. J. Nutr.* **2005**, *93*, 393–402. [CrossRef] [PubMed]
176. Demark-Wahnefried, W.; Polascik, T.J.; George, S.L.; Switzer, B.R.; Madden, J.F.; Ruffin, M.T.T.; Snyder, D.C.; Owzar, K.; Hars, V.; Albala, D.M.; et al. Flaxseed supplementation (not dietary fat restriction) reduces prostate cancer proliferation rates in men presurgery. *Cancer Epidemiol. Prev. Biomark.* **2008**, *17*, 3577–3587. [CrossRef] [PubMed]
177. Alphonse, P.; Aluko, R. A review on the anti-carcinogenic and anti-metastatic effects of flax seed lignan secolariciresinol diglucoside (SDG). *Discov. Phytomed.* **2015**, *2*, 12–17. [CrossRef]
178. Li, D.; Yee, J.A.; Thompson, L.U.; Yan, L. Dietary supplementation with secoisolariciresinol diglycoside (SDG) reduces experimental metastasis of melanoma cells in mice. *Cancer Lett.* **1999**, *142*, 91–96. [CrossRef]
179. Wang, L.; Chen, J.; Thompson, L.U. The inhibitory effect of flaxseed on the growth and metastasisof estrogen receptor negative human breast cancer xenograftsis attributed to both its lignan and oil components. *Int. J. Cancer* **2005**, *116*, 793–798. [CrossRef] [PubMed]
180. Pandima Devi, K.; Rajavel, T.; Daglia, M.; Nabavi, S.F.; Bishayee, A.; Nabavi, S.M. Targeting mirnas by polyphenols: Novel therapeutic strategy for cancer. *Semin. Cancer Biol.* **2017**, in press. [CrossRef] [PubMed]
181. Kozomara, A.; Griffiths-Jones, S. Mirbase: Integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* **2011**, *39*, D152–D157. [CrossRef] [PubMed]
182. Iorio, M.V.; Ferracin, M.; Liu, C.-G.; Veronese, A.; Spizzo, R.; Sabbioni, S.; Magri, E.; Pedriali, M.; Fabbri, M.; Campiglio, M. Microrna gene expression deregulation in human breast cancer. *Cancer Res.* **2005**, *65*, 7065–7070. [CrossRef] [PubMed]
183. Alvarez-Garcia, I.; Miska, E.A. MicroRNA functions in animal development and human disease. *Development (Cambridge, England)* **2005**, *132*, 4653–4662. [CrossRef] [PubMed]
184. Krol, J.; Loedige, I.; Filipowicz, W. The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet.* **2010**, *11*, 597–610. [CrossRef] [PubMed]
185. Metzler, M.; Wilda, M.; Busch, K.; Viehmann, S.; Borkhardt, A. High expression of precursor microrna-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer* **2004**, *39*, 167–169. [CrossRef] [PubMed]
186. Rana, T.M. Illuminating the silence: Understanding the structure and function of small RNAs. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 23–36. [CrossRef] [PubMed]
187. Lewis, B.P.; Burge, C.B.; Bartel, D.P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microrna targets. *Cell* **2005**, *120*, 15–20. [CrossRef] [PubMed]
188. Calin, G.A.; Sevignani, C.; Dumitru, C.D.; Hyslop, T.; Noch, E.; Yendamuri, S.; Shimizu, M.; Rattan, S.; Bullrich, F.; Negrini, M. Human microrna genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 2999–3004. [CrossRef] [PubMed]
189. Calin, G.A.; Dumitru, C.D.; Shimizu, M.; Bichi, R.; Zupo, S.; Noch, E.; Aldler, H.; Rattan, S.; Keating, M.; Rai, K. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15524–15529. [CrossRef] [PubMed]
190. Cimmino, A.; Calin, G.A.; Fabbri, M.; Iorio, M.V.; Ferracin, M.; Shimizu, M.; Wojcik, S.E.; Aqeilan, R.I.; Zupo, S.; Dono, M. MiR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 13944–13949. [CrossRef] [PubMed]
191. Michael, M.Z.; O'Connor, S.M.; van Holst Pellekaan, N.G.; Young, G.P.; James, R.J. Reduced accumulation of specific microRNAs in colorectal neoplasia11note: Susan M. O'connor and Nicholas G. Van Holst Pellekaan contributed equally to this work. *Mol. Cancer Res.* **2003**, *1*, 882–891. [PubMed]
192. Chen, C. Micrornas as oncogenes and tumor suppressors. *N. Engl. J. Med.* **2005**, *353*, 1768. [CrossRef] [PubMed]
193. Zaravinos, A. The regulatory role of microRNAs in EMT and cancer. *J. Oncol.* **2015**, *2015*, 865816. [CrossRef] [PubMed]

194. Gregory, P.A.; Bert, A.G.; Paterson, E.L.; Barry, S.C.; Tsykin, A.; Farshid, G.; Vadas, M.A.; Khew-Goodall, Y.; Goodall, G.J. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting Zeb1 and Sip1. *Nat. Cell Biol.* **2008**, *10*, 593–601. [CrossRef] [PubMed]
195. Park, S.-M.; Gaur, A.B.; Lengyel, E.; Peter, M.E. The mir-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors Zeb1 and Zeb2. *Genes Dev.* **2008**, *22*, 894–907. [CrossRef] [PubMed]
196. Kumarswamy, R.; Mudduluru, G.; Ceppi, P.; Muppala, S.; Kozlowski, M.; Niklinski, J.; Papotti, M.; Allgayer, H. MicroRNA-30A inhibits epithelial-to-mesenchymal transition by targeting snai1 and is downregulated in non-small cell lung cancer. *Int. J. Cancer* **2012**, *130*, 2044–2053. [CrossRef] [PubMed]
197. Korpal, M.; Ell, B.J.; Buffa, F.M.; Ibrahim, T.; Blanco, M.A.; Celià-Terrassa, T.; Mercatali, L.; Khan, Z.; Goodarzi, H.; Hua, Y. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. *Nat. Med.* **2011**, *17*, 1101–1108. [CrossRef] [PubMed]
198. Ma, L.; Young, J.; Prabhala, H.; Pan, E.; Mestdagh, P.; Muth, D.; Teruya-Feldstein, J.; Reinhardt, F.; Onder, T.T.; Valastyan, S. MiR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat. Cell Biol.* **2010**, *12*, 247–256. [CrossRef] [PubMed]
199. Martello, G.; Rosato, A.; Ferrari, F.; Manfrin, A.; Cordenonsi, M.; Dupont, S.; Enzo, E.; Guzzardo, V.; Rondina, M.; Spruce, T. A microrna targeting dicer for metastasis control. *Cell* **2010**, *141*, 1195–1207. [CrossRef] [PubMed]
200. Kong, W.; Yang, H.; He, L.; Zhao, J.-J.; Coppola, D.; Dalton, W.S.; Cheng, J.Q. MicroRNA-155 is regulated by the transforming growth factor β/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol. Cell. Biol.* **2008**, *28*, 6773–6784. [CrossRef] [PubMed]
201. Zhang, J.; Ma, L. Microrna control of epithelial–mesenchymal transition and metastasis. *Cancer Metastasis Rev.* **2012**, *31*, 653–662. [CrossRef] [PubMed]
202. Milenkovic, D.; Deval, C.; Gouranton, E.; Landrier, J.-F.; Scalbert, A.; Morand, C.; Mazur, A. Modulation of mirna expression by dietary polyphenols in apoE deficient mice: A new mechanism of the action of polyphenols. *PLoS ONE* **2012**, *7*, e29837. [CrossRef] [PubMed]
203. Li, Y.; VandenBoom, T.G., 2nd; Kong, D.; Wang, Z.; Ali, S.; Philip, P.A.; Sarkar, F.H. Up-regulation of mir-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer research* **2009**, *69*, 6704–6712. [CrossRef] [PubMed]
204. Hsieh, T.C.; Wu, J.M. Targeting CWR22Rv1 prostate cancer cell proliferation and gene expression by combinations of the phytochemicals EGCG, genistein and quercetin. *Anticancer Res.* **2009**, *29*, 4025–4032. [PubMed]
205. Saha, A.; Kuzuhara, T.; Echigo, N.; Suganuma, M.; Fujiki, H. New role of (−)-epicatechin in enhancing the induction of growth inhibition and apoptosis in human lung cancer cells by curcumin. *Cancer Prev. Res.* **2010**, *3*, 953–962. [CrossRef] [PubMed]
206. Niedzwiecki, A.; Roomi, M.W.; Kalinovsky, T.; Rath, M. Anticancer efficacy of polyphenols and their combinations. *Nutrients* **2016**, *8*, 552. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Development of Phenol-Enriched Olive Oil with Phenolic Compounds Extracted from Wastewater Produced by Physical Refining

Francesca Venturi [1,2,*], Chiara Sanmartin [1,2], Isabella Taglieri [1], Anita Nari [1], Gianpaolo Andrich [1,2], Erika Terzuoli [3], Sandra Donnini [3], Cristiano Nicolella [4,5] and Angela Zinnai [1,2]

1 Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy; chiara.sanmartin@unipi.it (C.S.); isabella.taglieri@for.unipi.it (I.T.); anita.nari@agr.unipi.it (A.N.); gianpaolo.andrich@unipi.it (G.A.); angela.zinnai@unipi.it (A.Z.)
2 Interdepartmental Research Center Nutrafood-Nutraceuticals and Food for Health, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy
3 Department of Life Science, University of Siena, Via Aldo Moro, 2, 53100 Siena, Italy; terzuoli8@unisi.it (E.T.); sandra.donnini@unisi.it (S.D.)
4 Department of Civil and Industrial Engineering, University of Pisa, Largo Lucio Lazzarino, 56122 Pisa, Italy; cristiano.nicolella@unipi.it
5 Consorzio Polo Tecnologico Magona, Via Magona, 57023 Cecina (LI), Italy
* Correspondence: francesca.venturi@unipi.it; Tel.: +39-050-221-6625

Received: 8 June 2017; Accepted: 16 August 2017; Published: 22 August 2017

Abstract: While in the last few years the use of olive cake and mill wastewater as natural sources of phenolic compounds has been widely considered and several studies have focused on the development of new extraction methods and on the production of functional foods enriched with natural antioxidants, no data has been available on the production of a phenol-enriched refined olive oil with its own phenolic compounds extracted from wastewater produced during physical refining. In this study; we aimed to: (i) verify the effectiveness of a multi-step extraction process to recover the high-added-value phenolic compounds contained in wastewater derived from the preliminary washing degumming step of the physical refining of vegetal oils; (ii) evaluate their potential application for the stabilization of olive oil obtained with refined olive oils; and (iii) evaluate their antioxidant activity in an in vitro model of endothelial cells. The results obtained demonstrate the potential of using the refining wastewater as a source of bioactive compounds to improve the nutraceutical value as well as the antioxidant capacity of commercial olive oils. In the conditions adopted, the phenolic content significantly increased in the prototypes of phenol-enriched olive oils when compared with the control oil.

Keywords: refining wastewater; enriched olive oil; antioxidant capacity; phenols; tyrosol; hydroxytyrosol; in vitro model; endothelial cells; cardiovascular diseases; cancer diseases

1. Introduction

The Mediterranean diet, where olive oil (OO) is the main source of fat, has been shown to reduce the incidence of age-associated diseases, including cardiovascular diseases, cancer, and neurodegenerative diseases [1]. Olive oil contains many bioactive components apart from oleic acid, including polyphenols. In preclinical and clinical studies, polyphenols have been reported to be responsible for some of the properties of olive oil, including anti-atherogenic, anti-inflammatory, anti-aging, anti-tumor, anti-viral, and immune modulator activities [1–3]. In 2011, the European Food Safety Authority (EFSA) endorsed a claim regarding the effectiveness of olive oil polyphenols

(5 mg/day) in protecting low-density lipoprotein (LDL) from oxidation [4], resulting in a significant anti-atherogenic effect [5].

According to European Union legislation [6], olive oil is classified into categories reflecting its quality and organoleptic properties, namely extra virgin olive oil (EVOO), virgin olive oil (VOO), lampante virgin olive oil (LVOO), refined olive oil (ROO) and also olive oil (OO), among others [7].

In particular, ROO is a low-quality oil that undergoes chemical or physical intervention to become edible, as it is usually obtained from VOO mechanically extracted from damaged olive fruits or from olives stored in unsuitable conditions and using refining methods. It has free acidity, expressed as oleic acid, of not more than 0.3 g per 100 g of oil [8], and is gaining importance in the food industry [7]. Further, ROO has a very reduced content of polyphenols since these compounds are among the substances eliminated during the refining process [9], so it is therefore unstable and subjected to rapid oxidation during storage [10].

As widely reported in the literature, oil manufacturers aim at producing foods that maintain their shelf life and nutritional quality over a defined period [11–13]. Thus, the use of antioxidants to minimize the oxidation of lipids in food materials is extensively accepted [14]. In this context, to overcome the stability problems of oils and fats, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) have been used as food additives [10]. However, recent reports reveal that these compounds may be implicated in many health risks, including cancer and carcinogenesis [15,16]. Due to these safety concerns, there is an increasing trend among food scientists to replace these synthetic antioxidants with natural ones, which are generally supposed to be safer [17,18].

In the last few years, the use of olive cake and mill wastewater as a natural source of phenolic compounds has been widely considered, and several studies have focused on the development of new extraction methods [11,13], as well as on the production of functional foods enriched with natural antioxidants [18,19]. In particular, oil-in-water emulsions formulated with stabilizers and enriched with phenolic compounds extracted from olive mill wastewater have recently been studied for the realization of emulsion-based food products with enhanced health properties [20,21]. However, to the best of our knowledge, no data are available in the literature on the feasibility of the production of a phenol-enriched refined olive oil using its own phenolic compounds extracted from wastewater produced during physical refining.

In this context, this study had three main objectives: (i) to verify the effectiveness of a multi-step extraction process to recover the high-added-value phenolic compounds potentially contained in wastewater produced during the preliminary washing degumming step of physical refining of vegetal oils; (ii) to evaluate their potential application for the stabilization of olive oil obtained with refined olive oils; and (iii) to evaluate their antioxidant activity in an in vitro model of endothelial cells.

2. Materials and Methods

2.1. Samples

The control olive oil (COO) was utilized as a matrix to carry out phenolic enrichment, and was obtained by mixing virgin olive oil (VOO, 7%) with refined olive oil (ROO, 93%), produced by a physical refining process at the industrial plant for vegetal oil refining managed by SALOV S.p.A. (Massarosa, Lucca, Italy).

Generally speaking, physical refining (also known as steam refining) is an industrial continuous process of deacidification and refining of crude oils as an alternative to alkali (chemical) neutralization [22]. In the proposed experimental protocol, the phenolic extracts were obtained from the wastewater taken at the physical refining plant outlet after the preliminary water degumming step before the steam distillation.

Both COO and wastewater samples were immediately stored at −20 °C in an inert atmosphere (N_2) to avoid oxidative damage.

As reported previously in Reference [11], the general chemical parameters (free acidity (% of oleic acid), peroxide value (meq O_2/kg), and K_{270}) of the starting COO as well as of the wastewater and phenol-enriched oil were determined at the Laboratory of Food Technology of DAFE (University of Pisa, Italy) according the analytical methods described in Regulation 2568/1991 of the European Union Commission and later modifications. The chemical composition of the starting COO and wastewater are reported in Table 1.

Table 1. Chemical composition control oil (COO) and wastewater (WW).

Parameter	Control Olive Oil (COO)	Wastewater (WW)
Free acidity (% of oleic acid)	0.12 ± 0.01	–
Peroxide value (meq O_2/kg)	5.46 ± 0.03	–
K_{270}	0.56 ± 0.03	–
pH	–	3.32 ± 0.02
Density	–	1.03 ± 0.01
Dry matter (d.m. %)	–	11.53 ± 0.10
Total phenol content (g/L of gallic acid)	0.025 ± 0.002	1.880 ± 0.004

2.2. Reagents

Phenolphthalein 1%, Folin–Ciocalteau reagent, and formaldehyde 40% (*m/v*) were purchased from Titolchimica (Pontecchio Polesine, Italy). Acetic acid, ethanol (99.8%), methanol, sodium carbonate anhydrous, diethyl ether, 2,2,4-trimethylpentane, hexane, hydrochloric acid 37%, sodium hydroxide 0.1 N, sodium thiosulphate 0.01 N, potassium persulfate, potassium iodine, chloroform, starch solution indicator 1%, as well as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), tyrosol, the standard of Trolox, tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), NaCl, NaF, H_2O_2, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and Triton were purchased from Sigma Aldrich (Milan, Italy). Hydroxytyrosol was obtained from Cayman Chemicals, Vinci Biochem (Vinci, Italy). DCFH2-DA (2,7-dichlorodihydrofluorescein diacetate) was purchased from Invitrogen (Milan, Italy). The standard gallic acid was supplied by Carlo Erba (Milan, Italy). Fetal calf serum was from EuroClone SpA (Milan, Italy). Diff-Quik was from Mertz-Dade AG, Dade International, (Milan, Italy), anti-caspase-3, anti-catalase and anti-SOD were from Cell Signaling, (Milan, Italy), secondary antibodies were from Promega (Padova, Italy), and cell culture dish and plates were from Sarstedt (Verona, Italy).

2.3. Preparation of Phenolic Extracts from Wastewater

To maximize the recovery of the phenolic compounds from wastewater collected during physical refining, we studied different extraction processes by utilizing two different solvent solutions: ethanol (99.8%), named sol. A; ethanol:diethyl ether (1:2 *v/v*), named sol. B.

In each extraction run, a sample of 25 mL of wastewater was rotary evaporated (Laborota 4000, Heidolph Instruments GMBH and Co. KG, Schwabach, Germany) until all water had been eliminated (150 rpm; 40 °C); the resulting extract was dissolved in 50 mL of extraction solution, shaken for 15 min, and then centrifuged (10,000 rpm (16,770× *g*), 5 min). The total phenol content of supernatant was determined spectrophotometrically at 280 nm (optical path = 1 mm), and calculations were performed using a calibration curve prepared with gallic acid as standard [23] for both solutions utilized for the extraction.

Finally, each extracted solution was rotary evaporated, and the resulting dry extract was stored at −20 °C under N_2 until its use in the oil enrichment. In these conditions, it was possible to store dry extracts, avoiding the addition of any antioxidant agent.

2.4. Preparation of Phenol-Enriched Oil

COO was used as a matrix enrichment by adding one of the wastewater extracts (WW-A, WW-B) to reach the maximum phenol concentration according to the chemical composition of the COO. Thus, two different phenol-enriched oils prototypes (PE-A and PE-B) were prepared. The extracts—obtained as described below—were incorporated into 25 mL of COO and kept shaking for 3 h in an inert atmosphere (N_2) in the dark at room temperature (20 ± 1 °C). Finally, the phenol-enriched oil prototypes were collected after centrifugation (IEC CL31R Multispeed, Thermo Scientific, Melegnano, Milan, Italy) at 10,000 rpm (16,770× *g*), 5 min, 15 °C, to eliminate any unsolved residues of dry extracts and maintained at 12 ± 1 °C until analysis.

2.5. Preparation of Phenolic Extracts from COO and Phenol-Enriched Oil

For the preparation of phenolic extracts from COO and phenol-enriched oil, we followed the method described by Montedoro et al. [24] with some modifications: liquid–liquid extraction with a solution of methanol:water (80:20 *v/v*) was carried out on the COO and phenol-enriched oil samples obtained as described before. In particular, 10 mL of oil samples were mixed with 10 mL of the mix solution, then the mixture was vigorously shaken for 3 min and, after 3 min of stopping in the dark, centrifuged for 15 min at 4000 rpm (2683× *g*). The phases were separated and the extraction was repeated successively two extra times. All the supernatant solutions obtained were stored at −20 °C under N_2 atmosphere overnight, until their use for total phenol content determination.

2.6. Total Phenol Content Determination (Wastewater, Phenolic Extracts from COO, and Phenol-Enriched Oils)

The total phenol content of the wastewater and phenolic extracts from COO and from phenol-enriched oils were determined colorimetrically at 765 nm, using the Folin–Ciocalteau reagent [25].

The total content of non-flavonoid phenolic compounds was determined according to the Kramling and Singleton method, as previously described [23].

Calculations were performed using a calibration curve prepared with gallic acid as the standard.

2.7. Bitter Index (BI)

In all samples (COO, PE-A, and PE-B), the bitter index was determined as reported by Gutierrez et al. [26]. Octadecyl (C_{18}) disposable extraction columns (6 mL) from J.T. Baker Chemical Company (Phillipsburg, NJ, USA) were used. For the extraction procedure of the bitter components, a sample of 1.0 ± 0.01 g virgin olive oil was dissolved in 4 mL hexane and passed over the C_{18} column, previously activated with methanol (6 mL) and washed with hexane (6 mL). After elution, 10 mL hexane was passed to eliminate the fat, and then the retained compounds were eluted with methanol:water (1:1) to 25 mL in a tared beaker. The absorbance of the extract was measured at 225 nm against methanol:water (1:1) in a 1 cm cuvette.

2.8. Antioxidant Capacity of the Olive Oils (COO and Phenol-Enriched Oils) by ABTS Assay

Antioxidant assay of methanolic extracts was performed following Sgherri et al. [27]. The radical cation ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) was generated as described by Pellegrini et al. [28]. The radical solution was diluted in water to obtain an absorbance at 734 nm of 0.70 ± 0.05. After 5 min from the addition of the extract (1% v/v), the decrease in absorbance was monitored and compared to that of the Trolox standard. The activities of the extracts were quantified by using a dose-response curve of Trolox in the 0.2–1.5 mM range, expressing them in terms of Trolox equivalent antioxidant capacity (TEAC) L^{-1} extract.

2.9. Cell Culture

Human umbilical cord vein endothelial cells (HUVEC) were from Cambrex and were maintained in basal Endothelial Growth Medium (EGM-2) and 10% fetal calf serum (FCS, Hyclone). Cells were split 1:3 twice a week, and used until they reached passage seven.

2.10. Cell Growth

The 1.5×10^3 cells resuspended in 10% fetal calf serum (FCS) were seeded in 96-multiwell plates. After adherence, cells were serum starved (0.1% FCS) for 24 h to synchronize cells, and then stimulated with test substances (25, 50, 100, and 200 μM hydrogen peroxide, H_2O_2, hydroxytyrosol, (HT), and tyrosol (Tyr), 0.1, 1, 10, 100 μM). After 48 h, cells were fixed in 100% methanol and stained with Diff-Quik. The total cell number/well was counted in a blinded manner at 10× magnification.

2.11. Reactive Oxygen Species (ROS) Measurement

Intracellular ROS was evaluated by a fluorimetric method. HUVEC cells (1.5×10^3 cells) were seeded in a 96-multiwell plate, and after adherence, were pre-treated with HT 10 μM and Tyr 10 μM, 30 min or 18 h, and then with H_2O_2 (100 μM, 90 min) in a medium without phenol red (for the concentration of HT and Tyr, please see Results Section 3.4). DCFH2-DA was added (10 μM, 30 min) and intracellular levels of ROS were evaluated with a microplate reader (excitation/emission 495/527) (Infinite 200 Pro SpectraFluor). Results are reported as relative fluorescence units (RFU) corrected for the cell number counted.

2.12. Western Blot

For Western blot analysis, 3×10^5 cells (HUVEC) were plated in 6 cm diameter dishes. After 24 h, cells were exposed to 1% FCS (Control condition, Ctr) or H_2O_2 in the presence or absence of polyphenols (HT 10 μM and Tyr 10 μM). Cells were scraped in a lysis buffer containing 50 mM of Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10 mM NaF, 1% Triton, and 1% protease inhibitor cocktail. Equal amounts (50 μg) of protein were separated by SDS-PAGE onto a gradient 4–12% gel and transferred to a nitrocellulose membrane. The membranes were blocked (1 h) in a solution of 5% (*w/v*) milk and then incubated overnight at 4 °C with the primary antibodies: anti-caspase-3, anti-catalase, and anti-superoxide dismutase (SOD) (each at 1:1000). After 1 h incubation in a secondary antibody anti-IgG horseradish peroxidase (HRP, diluted 1:2500), the immune reaction was revealed by a chemiluminescence system (ChemiDoc, BioRad, Milan, Italy). Results were normalized to those obtained by using an antibody against beta-actin or total caspase-3, when appropriate.

2.13. Statistical Analysis

To test the difference between the means among data sets (two replicates for each determination) One-way completely randomized ANOVA (CoStat, Cohort 6 software) was utilized. Comparisons among means were performed by the Bartlett's X2 corrected test ($p < 0.05$). Tukey's HSD multiple mean comparison test ($p < 0.05$) was used to state the differences among variables.

For biological analysis, results were either the representative or average of at least three independent experiments done in triplicate. Statistical analysis was performed using ANOVA test and *t*-test for unpaired data (Prism, GraphPad, La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Total Phenol Content of Wastewater and Wastewater Extracts

The total phenol content of wastewater as well as of the two phenolic extracts (WW-A and WW-B) obtained are reported in Table 2.

Table 2. Total phenol content of wastewater and wastewater extracts.

Sample	Total Phenol Content (g/L) as Gallic Acid	% of Non-Flavonoid Phenols
Wastewater (WW)	1.880 a	92%
WW Extract A (WW-A)	0.430 b	–
WW Extract B (WW-B)	0.292 c	–

Parameters not sharing the same letter have a significantly different mean concentration ($\alpha = 0.05$).

As seen in Table 2, the wastewater collected during olive oil physical refining exhibited very high levels of phenolic compounds, in most part represented by non-flavonoid compounds; therefore, they can be considered as a potential antioxidant source. In particular, the WW-A extract—obtained with pure ethanol (99.8%) as an extraction solution—showed the highest total phenolic amount.

3.2. Quality Parameters and Total Phenol Content of Enriched Control Olive Oils (COOs)

To verify the suitability of the proposed two prototypes of phenol-enriched oils (PE-A and PE-B), the total phenol content as well as the values of the general quality parameters (free acidity, peroxide value, and K_{270}) were evaluated (Table 3) and compared with those obtained from the COO.

Table 3. Quality parameters and total phenol content of control oil and phenol-enriched prototypes.

Sample	Free Acidity (% of Oleic Acid)	Peroxide Value (meq O_2/kg)	K_{270}	ΔK	K_{225}	Bitter Index (BI)	Total Phenol Content (g/kg of Gallic Acid)	% of Non-Flavonoid Phenols
COO	0.12 a	5.46 a	0.56 a	$\leq$0.15	0.10 c	0.52 c	0.025 c	99.4
PE-A	0.12 a	5.47 a	0.57 a	$\leq$0.15	0.15 b	1.20 b	0.105 b	74.9
PE-B	0.12 a	5.47 a	0.57 a	$\leq$0.15	0.18 a	1.52 a	0.131 a	100

Parameters not sharing the same letter have a significantly different mean concentration ($\alpha = 0.05$).

As seen, the values of the quality parameters determined in both phenol-enriched oils were within the range that makes them edible.

Furthermore, in the operating conditions adopted, the phenolic content significantly increased in both prototypes PE-A and PE-B compared with the COO and the values were assumed to appear close to those indicated for several extra-virgin olive oils labeled with protected geographical indication (PGI) [29].

In particular, the concentration of total phenols in the enriched oils was 4.2-fold greater than that determined in the starting COO when extract A (ethanol 99.8%) was used, while this value increased to reach 5.2 when extract B (ethanol:diethyl ether (1:2 *v*/*v*)) was used for the oil enrichment.

As shown in Table 3, the bitter index (BI, K_{225}) was directly proportional to the total phenolic content. Additionally, all samples analyzed could be classified as non-bitter or almost imperceptibly bitter oils, as they showed a value of $K_{225} \leq 0.25$, which was assumed as a reference point to indicate the appearance of some bitter taste by chemical analysis [26]. Further studies are needed to perform a proper panel test to confirm this evidence by sensory characterization.

3.3. Antioxidant Capacity of the Olive Oils (COO and Phenol-Enriched Oils) by ABTS Assay

The antioxidant capacity of the oils was analyzed by ABTS assay before (COO) and after (PE-A and PE-B) the enrichment. As shown in Figure 1, the antioxidant capacity of both the enriched oils was significantly higher than that shown by the control oil. In particular, the highest value of TEAC was determined for PE-B oil, which was characterized by the highest concentration of phenolic compounds, represented by non-flavonoid fraction (see Table 3).

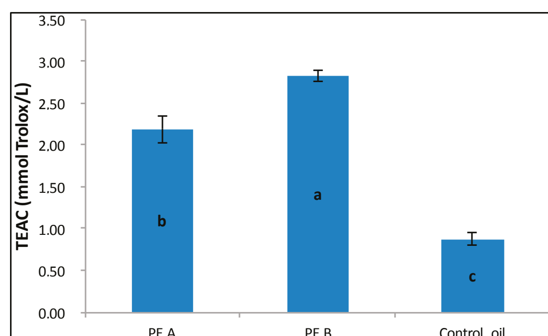


Figure 1. Trolox equivalent antioxidant capacity (TEAC) value determined for COO and both phenol-enriched olive oils. Parameters not sharing the same letter have a significantly different mean concentration (α = 0.05).

3.4. Polyphenols Recovery H_2O_2—Impaired Endothelial Cell Viability

The suspension of phenolic extracts in ethanol:diethyl ether (1:2 *v/v*) was not compatible with cell culture studies. Thus, for in vitro studies of phenolic extracts, the focus was on pure polyphenols. In both in vitro and in vivo studies, HT is the phenolic extract of olive oil that has been mainly investigated for its biological properties. HT has widely been considered to be beneficial for health, as it has been reported to prevent atherosclerosis and cancer, and to exhibit antimicrobial and anti-inflammatory activities [30–32]. Recently, it has been reported that HT inhibited colon cancer cell proliferation in vitro, and reduced tumor-mass growth in vivo in a mouse model [33]. Furthermore, it a possible mechanism has also been reported where HT increased the degradation of an epidermal growth factor receptor (an oncogenic signal in colon cancer) by promoting its ubiquitination [33]. Here, to investigate the properties of the polyphenols present in the phenolic extracts in a biological setting, the extracts were mimicked using a mix of HT and Tyr—the two major polyphenols of virgin olive oil [34]. The activity of the polyphenols was evaluated at concentrations between 0.1 and 100 μM in endothelial cell culture experiments, corresponding to nutritional/healthy-recommended polyphenol doses obtained from olive oil consumption for its anti-atherogenic activity [35]. First, it was investigated whether HT and Tyr would affect endothelial cell growth, per se and in combination. As shown in Table 4, HUVEC growth was unaffected by polyphenols at any concentration tested in the 48 h incubation. The effects exerted by the 100 μM concentration on cell viability were borderline toxic, and therefore in other experiments reported here, HT and Tyr were used at 10 μM, and served also for the mix. Next, the mix was tested for its effect on HUVEC growth, and as the combination of two compounds did not induce significant effects when compared with the control condition (1% FCS, Figure 2A), it was concluded that HT and Tyr—either per se or in combination (each at 10 μM)—did not affect endothelial viability.

Table 4. Endothelial cell number in response to polyphenols alone or in combination during 48 h culture.

Polyphenol Concentration Tested	Ctr	HT	Tyr	HT + Tyr
0	121 ± 4	—	—	—
0.1 μM	—	130 ± 7	123 ± 6	—
1 μM	—	126 ± 5	124 ± 4	—
10 μM	—	127 ± 5	125 ± 8	130 ± 8
100 μM	—	112 ± 6	108 ± 10	—

Data are reported as cells/well ± SEM (*n* = 4 run in triplicate). Polyphenols did not affect the number of endothelial cells in culture. Ctr = 1% fetal calf serum (FCS). Ctr: control; HT: hydroxytyrosol; Tyr: tyrosol.

Reactive oxygen species (ROS)—including hydrogen peroxide (H_2O_2) and superoxide radicals—have been reported as the cause of endothelial cell damage during atherosclerosis [30]. In particular, the endothelium has been reported to undergo apoptosis when exposed to ROS [36,37].

Thus, a combination of polyphenols was investigated for its effects on HUVEC growth when challenged with H_2O_2. Exposure of HUVEC to graded concentrations of H_2O_2 (25, 50, 100, and 200 μM) produced a significant decrease in cell count when compared to the control condition (1% FCS), demonstrating the sensitivity of HUVEC to ROS (Figure 2A). A selection of 100 μM H_2O_2 was made for further experiments since it inhibited cell viability by 60%. Co-incubation of HUVEC with the mix of polyphenols recovered endothelial cells from the damage produced by H_2O_2 (100 μM), indicating the protective effects of polyphenols on ROS-induced endothelium damage (Figure 2A). Given the reduction of cell number (suggestive of apoptosis produced by H_2O_2 treatment), we then measured the intracellular level of caspase-3 activity, long recognized as a reliable biochemical correlate of apoptotic events. Indeed, H_2O_2-induced apoptosis is known to be mediated through caspase-3 activation [31,36]. The results (shown in Figure 2B) demonstrated a significant increase of cleaved caspase-3 (the active form of caspase-3) in the HUVEC incubated with H_2O_2 for 6 h when compared with the control condition (1% FCS) ($p < 0.01$). The addition of the mix reversed the effect of H_2O_2 on caspase activity ($p < 0.01$) (Figure 2B).

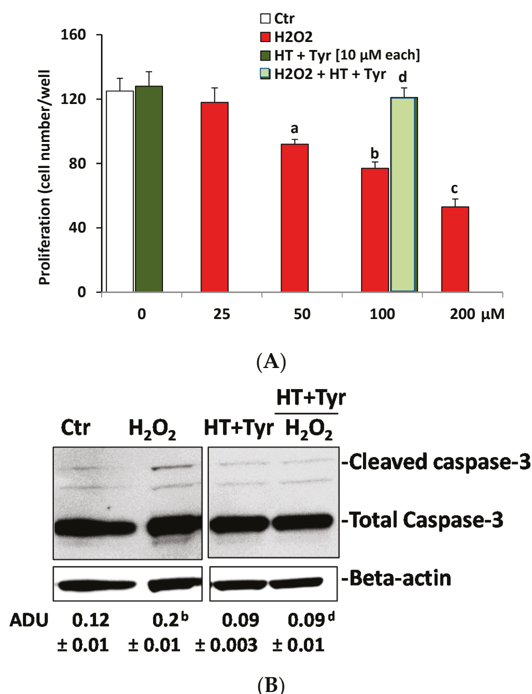


Figure 2. Polyphenols recover H_2O_2-induced HUVEC (human umbilical cord vein endothelial cells) proliferation and apoptosis. (**A**) HUVEC proliferation in response to H_2O_2 with/without HT + Tyr (48 h). Data are reported as cells/well ± SEM (*n* = 3 run in triplicate). Ctr = 1% FCS; [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ versus Ctr, [d] $p < 0.01$ versus H_2O_2 100 μM; (**B**) Cleaved caspase-3 in HUVEC exposed to H_2O_2 (100 μM) for 6 h, with/without HT + Tyr (10 μM each). Beta-actin was used for normalization. Total caspase-3 is shown as control of loading. Caspase-3 activity is expressed as arbitrary density unit (ADU). [b] $p < 0.01$ vs. 1% FCS, [d] $p < 0.01$ versus H_2O_2 100 μM. The gels shown are representative of three runs with similar results.

3.5. The Recovering Effects of Polyphenols are Linked to the Expression of Antioxidant Enzymes in Endothelium

Next it was analyzed whether the recovering effects of polyphenols on HUVEC viability was linked to their direct antioxidant activity or to other biological effects. To verify this hypothesis, first ROS production was measured in HUVEC pre-treated with the mix of polyphenols for 30 min or 18 h and then stimulated with H_2O_2 (100 µM, in 0.1% FCS) for 90 min by monitoring the oxidation of intracellular 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). ROS production was increased in treated cells compared to either low serum condition (0.1% FCS), control condition (1% FCS), or optimal serum condition (10% FCS), and this effect was significantly reduced in HUVEC pre-treated with polyphenols, either for 30 min or for 18 h (Figure 3A,B), suggesting both a direct and indirect antioxidant activity of polyphenols.

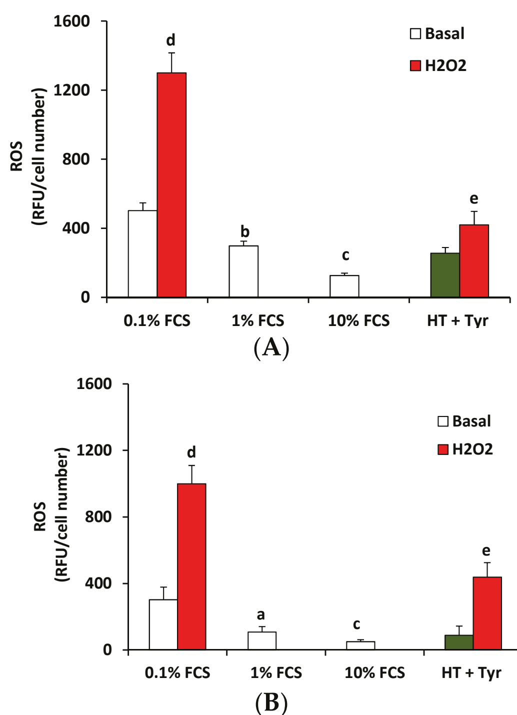


Figure 3. The HT + Tyr combination decreases reactive oxygen species (ROS) levels in HUVEC exposed to H_2O_2. HUVEC were stimulated with HT + Tyr (10 µM each, green bar) in 0.1% FCS for (**A**) 30 min or (**B**) 18 h and then exposed to H_2O_2 (100 µM, 90 min). Data are expressed as relative fluorescent units (RFU)/cell number (n = 3). ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$ vs. 0.1% FCS, ^{d} $p < 0.001$ vs. 0.1% FCS, ^{e} $p < 0.001$ vs. H_2O_2.

Next, it was investigated whether the polyphenols' inhibitory activity on ROS levels at 18 h was mediated by the recovering levels of antioxidant-related signals (SOD and catalase) in endothelial cells. It was found that 100 µM H_2O_2 decreased SOD and catalase expression at 18 h (Figure 4). The mix of polyphenols (which had a mild activity per se) in combination with 100 µM H_2O_2 recovered SOD and catalase expression when compared with the control condition (1% FCS), suggesting that the long-lasting protective effects of polyphenols on cell damage induced by ROS might be linked to their indirect antioxidant properties.

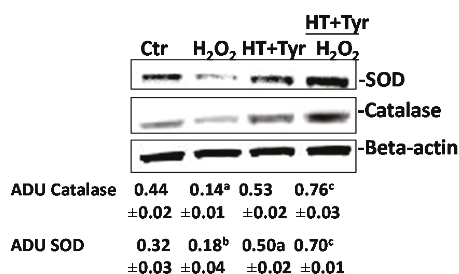


Figure 4. Combination of polyphenols rescues superoxide dismutase (SOD) and catalase expression in HUVEC exposed to H_2O_2. Western blot analysis of SOD and catalase in HUVEC exposed to H_2O_2 (100 μM) with/without HT + Tyr (10 μM each). Beta-actin is used for normalization. Expression of catalase or SOD is reported as arbitrary density unit (ADU). ^{a} $p < 0.05$, ^{b} $p < 0.01$ vs. 1% Ctr = FCS; ^{c} $p < 0.01$ versus H_2O_2. The gels are representative of three runs with similar results.

Together, the in vitro findings showed that the mix of HT and Tyr significantly prevented the damage mediated by hydrogen peroxide, sustaining endothelial cell growth and the expression of antioxidant enzymes such as superoxide dismutase and catalase. Specifically, the combination of HT and Tyr prevented endothelial cells from entering the H_2O_2-induced apoptosis, restoring their viability and their inherent capacity to proliferate in response to serum (1% FCS). In the endothelial cells (known for their sensitivity to the external environment), the mechanism of the anti-apoptotic effect exerted by the mix HT plus Tyr appeared to be either dependent or independent of scavenging activity, and the mix of polyphenols provided a survival advantage capable of overriding the cytotoxic insult. In this context, HT and Tyr appeared to be alternative agents to the known purely scavenger molecules in reducing oxidative stress-induced vascular damage. The knowledge that these small molecules could prevent the persistent damaging effects of ROS might have implications for the design of novel therapies for cardiovascular pathologies where the dysfunction of the endothelium was the underlying causative factor.

These findings, together with the effects of HT on cancer cells, may provide a rational mechanistic framework for the health benefits reported in epidemiological studies on the Mediterranean diet.

4. Conclusions

At laboratory scale, different extraction solutions were tested to develop an extraction method to recover high added-value compounds from the wastewater produced during the physical refining of olive oil. The extracts were then evaluated for their potential application in the phenol-enrichment and stabilization of commercial olive oils.

The results showed that wastewater collected during the physical refining process could be considered as a good source of bioactive compounds useful for a significant increase of nutraceutical value, as well as of the antioxidant capacity of olive oils.

Currently, many studies on olive oil-mediated beneficial health effects have indicated that it reduced oxidation of the low-density lipoprotein carrying cholesterol (LDL-C), and inhibited thrombogenic events [38–40]. In agreement with these experimental observations, epidemiological and clinical studies have reported that olive oil reduced the incidence of cardiovascular diseases [41,42]. In this study, in a model of in vitro endothelial cells, the combination of HT with Tyr—the two major olive oil polyphenols which mimic the phenolic extracts from COO—when used at the recommended concentration from EVOO consumption, preserved cell functions from oxidative damage, rescuing their antioxidant properties.

Further studies are needed to investigate the protective properties of phenolic extracts in enriched olive oil, as well as to validate the proposed method and to explore the feasibility of its industrial

scale-up. In this context, close attention should be paid to the following main aspects: the evaluation of the chemical composition of the phenol-enriched olive oils (PE-A and PE-B); the determination of the possible residue of solvents in the industrial enriched oil; the setup and standardization of the industrial process; the evaluation of the cost effectiveness of the industrial production; and the quantification of the added value that could be assigned to the new product.

Acknowledgments: This work was supported by Regione Toscana, bando Nutraceutica 2014 (DD650/2014) OlivaPlus project and bando Agrifood Praf 2012-2015 (Mis 1.2.e) Nutriforoil project. This research project was made possible by the collaboration with SALOV Group located in Massarosa (LU, Italy) and with Consorzio Polo Tecnologico Magona located in Cecina (LI, Italy). Mike F. Quartacci and Cristina Sgherri are acknowledged for their assistance during antioxidant assay of methanolic extracts.

Author Contributions: Authors affiliated to DAFE, University of Pisa were involved in the development of phenol-enriched olive oil and in the evaluation of their antioxidant activity, while authors affiliated to DSV, University of Siena, were involved in the evaluation of polyphenols effects in an in vitro model of endothelial cells. Francesca Venturi, Angela Zinnai, Sandra Donnini and Cristiano Nicolella conceived and designed the experiments; Chiara Sanmartin, Isabella Taglieri, Anita Nari and Erika Terzuoli performed the experiments; Chiara Sanmartin, Gianpaolo Andrich and Erika Terzuoli analyzed the data; and Francesca Venturi and Sandra Donnini wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. López-Miranda, J.; Pérez-Jiménez, F.; Ros, E.; De Caterina, R.; Badimón, L.; Covas, M.I.; Escrich, E.; Ordovás, J.M.; Soriguer, F.; Abiá, R.; et al. Olive oil and health: Summary of the II international conference on olive oil and health consensus report, Jaén and Córdoba (Spain) 2008. *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 284–294. [CrossRef] [PubMed]
2. Covas, M.I. Olive oil and cardiovascular system. *Pharmacol. Res.* **2007**, *55*, 175–186. [CrossRef] [PubMed]
3. Fitó, M.; De la Torre, R.; Covas, M.I. Olive oil and oxidative stress. *Mol. Nutr. Food Res.* **2007**, *51*, 1215–1224. [CrossRef] [PubMed]
4. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific Opinion on the substantiation of health claims related to polyphenols in olive oil and protection of LDL particles from oxidative damage. *EFSA J.* **2011**, *9*, 2033–2058.
5. Valls, R.M.; Farràs, M.; Suárez, M.; Fernández-Castillejo, S.; Fitó, M.; Konstantinidou, V.; Fuentes, F.; López-Miranda, J.; Giralt, M.; Covas, M.I.; et al. Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomized controlled trial. *Food Chem.* **2015**, *167*, 30–35. [CrossRef] [PubMed]
6. European Communities (EC). Commission Regulation (EC) No. 1989/2003 of 6 November 2003. *Off. J. Eur. Commun.* **2003**, *295*, 57–77.
7. Garcia, R.; Martins, N.; Cabrita, M.J. Putative markers of adulteration of extra virgin olive oil with refined olive oil: Prospects and limitations. *Food Res. Int.* **2013**, *54*, 2039–2044. [CrossRef]
8. International Olive Oil Council (IOOC). *Trade Standard Applying to Olive Oils and Olive-Pomace Oils 2011*; COI/T.15/NC No. 3/Rev; 6 November 2011; International Olive Oil Council: Madrid, Spain, 2011; pp. 1–17. Available online: http://www.internationaloliveoil.org/estaticos/view/222-standards (accessed on 8 June 2017).
9. Garcìa, A.; Ruiz-Mendez, M.V.; Romero, C.; Brenes, M. Effect of refining on the phenolic composition of crude olive oils. *J. Am. Oil Chem. Soc.* **2006**, *83*, 159–164. [CrossRef]
10. Bouaziz, M.; Fki, I.; Jemai, H.; Ayadi, M.; Sayadi, S. Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves. *Food Chem.* **2008**, *108*, 253–262. [CrossRef]
11. Zinnai, A.; Venturi, F.; Sanmartin, C.; Taglieri, I.; Andrich, G. The utilization of solid carbon dioxide in the extraction of extra-virgin olive oil. *Agro Food Ind. Hi Tech.* **2015**, *26*, 24–26.
12. Zinnai, A.; Sanmartin, C.; Taglieri, I.; Andrich, G.; Venturi, F. Supercritical fluid extraction from microalgae with high content of LC-PUFAs. A case of study: Sc-CO_2 oil extraction from *Schizochytrium* sp. *J. Supercrit. Fluids* **2016**, *116*, 126–131. [CrossRef]

13. Venturi, F.; Sanmartin, C.; Taglieri, I.; Andrich, G.; Zinnai, A. A simplified method to estimate Sc-CO_2 extraction of bioactive compounds from different matrices: Chili pepper vs. tomato by-products. *Appl. Sci.* **2017**, *7*, 361. [CrossRef]
14. Fki, I.; Allouche, N.; Sayadi, S. The use of polyphenolic extract, purified hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid from olive mill wastewater for the stabilization of refined oils: A potential alternative to synthetic antioxidants. *Food Chem.* **2005**, *93*, 197–204. [CrossRef]
15. Hou, D.X. Potential mechanisms of cancer hemoprevention by anthocyanins. *Curr. Mol. Med.* **2003**, *3*, 149–159. [CrossRef] [PubMed]
16. Prior, R.L. Absorption and metabolism: Potential health effect. In *Phytochemicals: Mechanism of Action*, 1st ed.; Meskin, M., Bidlack, W.R., Davies, A.J., Lewis, D.S., Randolph, R.K., Eds.; CRC Press: Boca Raton, FL, USA, 2003; pp. 1–18.
17. Yanishlieva, N.V.; Marinova, E.M. Stabilization of edible oils with natural antioxidants. *Eur. J. Lipid Sci. Tech.* **2001**, *103*, 752–767. [CrossRef]
18. Suárez, M.; Romero, M.P.; Motilva, M.J. Development of a phenol enriched olive oil with phenolic compounds from olive cake. *J. Agric. Food Chem.* **2010**, *58*, 10396–10403. [CrossRef] [PubMed]
19. Caporaso, N.; Formisano, D.; Genovese, A. Use of phenolic compounds from olive mill wastewater as valuable ingredients for functional foods. *Crit. Rev. Food Sci. Nutr.* **2017**. [CrossRef] [PubMed]
20. Caporaso, N.; Genovese, A.; Burke, R.; Barry-Ryan, C.; Sacchi, R. Physical and oxidative stability of functional olive oil-in-water emulsions formulated using olive mill wastewater biophenols and whey proteins. *Food Funct.* **2016**, *7*, 227–238. [CrossRef] [PubMed]
21. Caporaso, N.; Genovese, A.; Burke, R.; Barry-Ryan, C.; Sacchi, R. Effect of olive mill wastewater phenolic extract, whey protein isolate and xanthan gum on the behavior of olive O/W emulsions using response surface methodology. *Food Hydrocoll.* **2016**, *61*, 66–76. [CrossRef]
22. Tzia, C.; Giannou, V.; Polychniatou, V.; Chanioti, S. Fat and oil processing technology. In *Handbook of Food Processing: Food Safety, Quality, and Manufacturing Processes*, 1st ed.; Varzakas, T., Tzia, C., Eds.; CRC Press: Boca Raton, FL, USA, 2015; pp. 381–424.
23. Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* **1992**, *40*, 1571–1578. [CrossRef]
24. Zinnai, A.; Venturi, F.; Andrich, G. Time evolution of phenols extractions from Sangiovese grapes with and without the addition of solid carbon dioxide. *Agrochimica* **2011**, *55*, 193–202.
25. Folin, O.; Ciocalteau, V. On tyrosine and tryptophan determination in protein. *J. Biol. Chem.* **1927**, *73*, 627–650.
26. Gutiérrez Rosales, F.; Perdiguero, S.; Gutierrez, R.; Olias, J.M. Evaluation of the bitter taste in virgin olive oil. *J. Am. Oil Chem. Soc.* **1992**, *69*, 394–395. [CrossRef]
27. Sgherri, C.; Micaelli, F.; Andreoni, N.; Baldanzi, M.; Ranieri, A. Retention of phenolic compounds and antioxidant properties in potato bread obtained from a dough enriched with a powder from the purple cv. Vitelotte. *Agrochimica* **2016**, *60*, 312–328. [CrossRef]
28. Pellegrini, N.; Re, R.; Yang, M.; Rice-Evans, C. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2′-azinobis[3-ethylenebenzothiazoline-6-sulfonic acid] radical cation decolorization assay. *Methods Enzymol.* **1999**, *299*, 379–389. [CrossRef]
29. D.M. 21 July 1998. Production Specifications for Protected Geographical Indication of "Toscano" Extra Virgin Olive Oil. Gazz. Uff. N. 243. 17 October 1998. Available online: http://www.oliotoscanoigp.it/cms/doc/DisciplinareOlioToscanoIGP.pdf (accessed on 8 June 2017).
30. Irani, K. Oxidant signaling in vascular cell growth, death and survival. A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ. Res.* **2000**, *87*, 179–183. [CrossRef] [PubMed]
31. De la Puerta, R.; Ruiz Gutierrez, V.; Hoult, J.R. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* **1999**, *57*, 445–449. [CrossRef]
32. Tripoli, E.; Giammanco, M.; Tabacchi, G.; Di Majo, D.; Giammanco, S.; La Guardia, M. The phenolic compounds of olive oil: Structure, biological activity and beneficial effects on human health. *Nutr. Res. Rev.* **2005**, *18*, 98–112. [CrossRef] [PubMed]

33. Terzuoli, E.; Giachetti, A.; Ziche, M.; Donnini, S. Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation. *Mol. Nutr. Food Res.* **2016**, *60*, 519–529. [CrossRef] [PubMed]
34. Corona, G.; Tzounis, X.; Assunta Dessì, M.; Deiana, M.; Debnam, E.S.; Visioli, F.; Spencer, J.P. The fate of olive oil polyphenols in the gastrointestinal tract: Implications of gastric and colonic microflora-dependent biotransformation. *Free Radic. Res.* **2006**, *40*, 647–658. [CrossRef] [PubMed]
35. Fernández-Bolaños, J.G.; López, O.; Fernández-Bolanos, J.; Rodríguez-Gutiérrez, G. Hydroxytyrosol and derivatives: Isolation, synthesis, and biological properties. *Curr. Org. Chem.* **2008**, *12*, 442–463. [CrossRef]
36. Cantara, S.; Donnini, S.; Giachetti, A.; Thorpe, P.E.; Ziche, M. Exogenous BH4/Bcl-2 peptide reverts coronary endothelial cell apoptosis induced by oxidative stress. *J. Vasc. Res.* **2004**, *41*, 202–207. [CrossRef] [PubMed]
37. Le Bras, M.; Clement, M.V.; Pervaiz, S.; Brenner, C. Reactive oxygen species and the mitochondrial signaling pathway of cell death. *Histol. Histopathol.* **2005**, *20*, 205–219. [CrossRef] [PubMed]
38. Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Del Carlo, M.; Gallina-Toschi, T.; Lercker, G.; Compagnone, D.; Fernández-Gutiérrez, A. Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. *J. Agric. Food Chem.* **2005**, *53*, 8918–8925. [CrossRef] [PubMed]
39. Visioli, F.; Galli, C.; Galli, G.; Varuso, D. Biological activities and metabolic fate of olive oil phenols. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 677–684. [CrossRef]
40. Morbidelli, L. Polyphenol-based nutraceuticals for the control of angiogenesis: Analysis of the critical issues for human use. *Pharmacol. Res.* **2016**, *111*, 384–393. [CrossRef] [PubMed]
41. Estruch, R.; Ros, E.; Salas-Salvadó, J.; Covas, M.I.; Corella, D.; Arós, F.; Gómez-Gracia, E.; Ruiz-Gutiérrez, V.; Fiol, M.; Lapetra, J.; et al. Primary prevention of cardiovascular disease with a Mediterranean diet. *N. Engl. J. Med.* **2013**, *368*, 1279–1290. [CrossRef] [PubMed]
42. Monti, M.; Terzuoli, E.; Ziche, M.; Morbidelli, L. The sulphydryl containing ACE inhibitor Zofenoprilat protects coronary endothelium from Doxorubicin-induced apoptosis. *Pharmacol. Res.* **2013**, *76*, 171–181. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Strawberries Improve Pain and Inflammation in Obese Adults with Radiographic Evidence of Knee Osteoarthritis

Jace Schell [1], R. Hal Scofield [2,3,4], James R. Barrett [5], Biji T. Kurien [2], Nancy Betts [1], Timothy J. Lyons [6], Yan Daniel Zhao [7] and Arpita Basu [1,8,*]

1 Nutritional Sciences, Oklahoma State University, Stillwater, OK 74078, USA; jace.schell@okstate.edu (J.S.); nancy.betts@okstate.edu (N.B.)
2 Section of Endocrinology and Diabetes, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA; Hal-Scofield@omrf.org (R.H.S.); biji-kurien@omrf.org (B.T.K.)
3 Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA
4 Medical Service, US Department of Veterans Affairs Medical Center, Oklahoma City, OK 73102, USA
5 Department of Family and Preventive Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA; jim-barrett@ouhsc.edu
6 Division of Endocrinology, Medical University of South Carolina, Charleston, SC 29425, USA; lyonstj@musc.edu
7 Biostatistics and Epidemiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA; Daniel-zhao@ouhsc.edu
8 Kinesiology and Nutrition Sciences, University of Nevada Las Vegas, Las Vegas, NV 89154, USA
* Correspondence: arpita.basu@unlv.edu; Tel.: +1-702-895-4576

Received: 22 July 2017; Accepted: 25 August 2017; Published: 28 August 2017

Abstract: Osteoarthritis (OA), the most common form of arthritis, is a significant public health burden in U.S. adults. Among its many risk factors, obesity is a key player, causing inflammation, pain, impaired joint function, and reduced quality of life. Dietary polyphenols and other bioactive compounds in berries, curcumin, and tea have shown effects in ameliorating pain and inflammation in OA, but few clinical studies have been reported. The purpose of the present study was to examine the effects of dietary strawberries on pain, markers of inflammation, and quality of life indicators in obese adults with OA of the knee. In a randomized, double-blind cross-over trial, adults with radiographic evidence of knee OA (n = 17; body mass index (BMI): (mean ± SD) 39.1 ± 1.5; age (years): 57 ± 7) were randomized to a reconstituted freeze-dried strawberry beverage (50 g/day) or control beverage daily, each for 12 weeks, separated by a 2-week washout phase (total duration, 26 weeks). Blood draws and assessments of pain and quality of life indicators were conducted using the Visual Analog Scale for Pain (VAS Pain), Measures of Intermittent and Constant Osteoarthritis Pain (ICOAP), and Health Assessment Questionnaire-Disability Index (HAQ-DI) questionnaires, which were completed at baseline and at weeks 12, 14, and 26 of the study. Among the serum biomarkers of inflammation and cartilage degradation, interleukin (IL)-6, IL-1β, and matrix metalloproteinase (MMP)-3 were significantly decreased after strawberry vs. control treatment (all $p < 0.05$). Strawberry supplementation also significantly reduced constant, intermittent, and total pain as evaluated by the ICOAP questionnaire as well as the HAQ-DI scores (all $p < 0.05$). No effects of treatment were noted on serum C-reactive protein (CRP), nitrite, glucose, and lipid profiles. Dietary strawberries may have significant analgesic and anti-inflammatory effects in obese adults with established knee OA.

Keywords: strawberries; knee osteoarthritis; pain; inflammation

1. Introduction

Osteoarthritis (OA), the most common type of arthritis, is a chronic, painful, and inflammatory musculoskeletal disease causing functional impairment in approximately 27 million Americans; obesity and advancing age are important risk factors [1,2]. While there is no cure, the current management of OA combines nonpharmacological and pharmacological interventions, and often involves costly joint replacement procedures [3]. Non-steroidal anti-inflammatory drugs may lead to gastrointestinal side-effects, and effective, safer alternatives could benefit millions of patients. Nutraceuticals are good candidates for the management of OA, due to their safety profile and potential efficacy. However, the popularly used supplements, such as glucosamine, chondroitin sulfate, and avocado–soy unsaponifiables, have failed to show a convincing and significant mitigation of symptoms in a meta-analysis of randomized clinical trials, or lack long-term studies on the clinical symptoms and biomarkers of knee OA [4–6].

Obesity is considered a significant risk factor for OA, and contributes to the chronic inflammation that underlies the pathogenesis and symptoms of the condition [7,8]. Biomarkers of inflammation, especially serum C-reactive protein (CRP) and interleukin-6 (IL-6), and those of cartilage degradation, including matrix metalloproteinases (MMPs), have been positively correlated with pain and the progression of OA [9–12]. Dietary bioactive compounds, such as curcumin [13,14], ginger [15], green tea polyphenols [16], and herbal tea [17] have been shown to be effective in the management of pain symptoms and in reducing inflammatory biomarkers of OA. However, such clinical studies are few in number and of short duration, mostly examine pain symptoms but not disease biomarkers, and involve non-obese and otherwise healthy participants. Among the nutritional supplements and foods containing bioactive compounds, polyphenol-rich dietary berries have been extensively studied for their protective associations with other chronic conditions, including hypertension [18], type 2 diabetes [19], and overall inflammation [20], as reported in epidemiological studies. Berries, such as blueberries and red raspberries, have been shown to reduce the symptoms and progression of arthritis, such as pain and articular degeneration, in collagen-induced experimental models of arthritis [21,22]. Our group has previously reported the safety and efficacy of berries, especially freeze-dried blueberries and strawberries, in improving features of the metabolic syndrome and decreasing surrogate biomarkers of atherosclerosis in clinical studies [23–25]. To our knowledge, no previous clinical study has been reported on the effects of berries on OA of the knee in adults.

For this reason, we undertook the present study to examine the effects of freeze-dried strawberries on pain symptoms and on circulating biomarkers of inflammation and cartilage degradation in obese adults with symptomatic knee OA. Our primary aim was to determine the effects of freeze-dried strawberries on pain scores assessed by the Visual Analog Scale for Pain (VAS Pain) and those based on a Measure of Intermittent and Constant Osteoarthritis Pain (ICOAP) survey, as well as on selected biomarkers of inflammation and cartilage degradation associated with knee OA in comparison to a control group.

2. Materials and Methods

2.1. Participants

Obese participants with a body mass index (BMI) >30 kg/m^2, a large waist circumference (>35 in for women and >40 in for men), and radiographic evidence of knee OA were enrolled in the study. A diagnosis of knee OA was verified by a rheumatologist based on the radiological evidence of mild to moderate bilateral primary knee OA defined by the American College of Rheumatology (ACR) [26]. Radiological evidence of degenerative OA, but without fractures or dislocation, confirmed eligibility for the study. Participants were excluded if they had any of the following conditions: previous knee surgery, rheumatoid arthritis, a metabolic disorder (such as diabetes and cancer), liver or kidney failure, pregnant or lactating, use of corticosteroids and/or intra-articular injections during the preceding 3 months, use of fish oils and glucosamine, participation in a weight loss program in the preceding 6 months, and recent changes in physical activity levels, regular smoking, or allergic to strawberries.

In addition, participants who were unable to express their pain (such as those with any mental condition) were also excluded from the study. The study (the ethics approval code: HE1517) was approved by the ethics committees at the University of Oklahoma Health Sciences Center (OUHSC) and at Oklahoma State University (OSU). All participants provided written informed consent prior to enrollment in the study. The trial was registered with clinicaltrials.gov (NCT02518347).

2.2. Study Design and Intervention

Participants were recruited at the Oklahoma Clinical and Translational Sciences Institute (OCTSI) at OUHSC and at the Department of Nutritional Sciences Clinical Assessment Unit at OSU. The recruitment was conducted though campus-wide e-mail advertisements and physician referrals. Upon qualification, the participants were randomly assigned to one of the two study groups in a 26-week crossover study: strawberry and control. Randomization was performed using a sequence of randomly generated numbers using SAS (Version 9.4; SAS Institute Inc., Cary, NC, USA). Each intervention was for 12 weeks, with and intervening two weeks of washout phase. During the active treatment phase, the participants consumed 50 g of freeze-dried strawberry powder reconstituted in water twice a day. This dose of strawberry powder is equivalent to approximately 500 g of fresh strawberries, and was previously used in another study [24]. The control powder was formulated to match the sensory properties of the strawberry powder as well as its caloric value and macronutrient composition. Table 1 shows the nutritional composition of the strawberry and control powders provided by the California Strawberry Commission (Watsonville, CA, USA). The nutrient and phytochemical composition of the strawberry and control powders was determined at the Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University (Stillwater, OK, USA), and at the Brunswick Laboratories (Southborough, MA, USA), respectively. The participants were asked to consume the strawberry or control beverage twice a day, at similar time points that were at least six to eight hours apart, and also to consume the beverage as a snack by itself, and not with a meal or other snacks to prevent the confounding effects of other dietary factors. The participants were instructed to take the last dose of the test beverage at least 10–12 h prior to the fasting blood draw the following morning. The participants were also asked to refrain from consuming other berry products during the study, and to maintain usual diet and physical activity. Compliance was assessed by the return of unused test agents and a mandatory three visits per week to the clinic for supervised consumption and a determination of plasma ellagic acid [27].

Table 1. Composition of the freeze-dried strawberry and control powders [1].

Nutrients/Compounds	Strawberry Powder	Control Powder
Dose, g	50	50
Calories, kcal	160	172
Carbohydrates, g	35	38
Total polyphenols, mg GAE	1585	75
Total anthocyanins, mg cyanidin-3-glucoside equivalents	66	-
Ellagic acid, mg	220	-
Phytosterols, mg	50	-
Dietary fiber, g	8	5

[1] Supplied by California Strawberry Commission (Watsonville, CA, USA); analysis conducted at the Robert M. Kerr Food and Agricultural Products Center at Oklahoma State University, polyphenol and anthocyanin analyzed by Brunswick Laboratories (Southborough, MA, USA); GAE: gallic acid equivalents.

2.3. Biochemical Variables

Freshly drawn blood samples were sent to the OU Medical Center laboratory for an analysis of a comprehensive metabolic panel, including serum glucose, lipid profiles, HbA1c, and high-sensitivity C-reactive protein (hs-CRP), using an automated clinical analyzer (Abbott Architect Instruments). Serum IL-6, IL-1β, and MMP-3 and 8 were measured using ELISA kits based on the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA) with inter-assay CVs (Coefficient of variation) of

4.5%, 7.6%, 3.6%, and 7.5%, respectively. Serum nitrite was measured using the Griess Reagent System (Promega Corporation, Madison, WI, USA) with a mean inter-assay CV of 3.3%.

2.4. Pain Scores and Quality of Life Indicators

Knee pain scores were assessed using the ICOAP survey, a multidimensional, OA-specific measure designed to provide a comprehensive evaluation of pain experience in people with knee OA, that has been used in several large studies [28,29]. The ICOAP is an 11-item scale evaluating two pain domains: a 5-item scale evaluating constant pain and a 6-item scale evaluating intermittent pain. We also used the VAS Pain and health scale to assess the visual perception of the participants' pain intensity and feeling of well-being [30]. In addition, the Health Assessment Questionnaire-Disability Index (HAQ-DI) was used to assess functional ability using 20 items distributed across eight dimensions (dressing, arising, eating, walking, reach, grip, hygiene, and daily activity), rating each according to a four-level disability scale (range 0–3) [30]. The participants were asked to fill out the questionnaires on the morning of their fasting blood draws visits, at least 10–12 h following their last test dose of strawberry and control beverage.

2.5. Dietary Analysis

Habitual intake of food and beverages was recorded using 3-day food records (two weekdays and one weekend day) at baseline, and at week 6, 12, 14, 20, and 26 of the study. At enrollment, the participants were educated to record food and beverages by a study Registered Dietitian (RD) using food models and utensils for the estimation of portion sizes. Nutrient intakes were analyzed using Nutritionist Pro version 3.2 (Axxya Systems LLC, Redmond, WA, USA). The averages of three days were used to estimate the nutrient intakes per week for each participant.

2.6. Statistical Analysis

For baseline demographics and characteristics, continuous variables were expressed as means ± SD and discrete variables were presented as counts and proportions. Our main objective was to assess whether the selected biomarkers of inflammation and cartilage degradation, as well as knee pain scores, were different between the strawberry and control phases at 12 vs. 26 weeks of the crossover study. To test this aim, we used a linear mixed-effects model (PROC MIXED) with time as within-subject factor and intervention group as a between-subject factor for each variable. Data were corrected for baseline values. We also examined associations of the serum biomarkers with knee pain scores at baseline using a multiple linear regression model, adjusting for baseline age, BMI, and energy intake. The assumptions used in the sample size calculation were conservative, based on the report by Panahi et al. [13]. From previous dietary intervention studies in knee OA, we expected a decrease in serum IL-6 in the range of 0.39–0.45 pg/mL [31]. All p-values < 0.05 were considered statistically significant and data were analyzed using SAS/STAT software (Version 9.4; SAS Institute Inc., Cary, NC, USA).

3. Results

Among the 35 participants who were screened, 17 qualified and completed the 26-week study (Figure 1). The baseline characteristics of these participants are shown in Table 2. There were no drop-outs in the study. Among the participants who completed the study, compliance was 100% for the strawberry group and 97% for the control group as assessed by mandatory thrice weekly visits, with the return of any unconsumed strawberry and control powder on the days the participants did not come to the clinic. No adverse events were reported in the study. As a measure of compliance, plasma ellagic acid was detectable in 17 participants in the strawberry phase (means ± SEMs (Standard error of means), 30.2 ± 3.6 ng/mL), whereas concentrations were not detectable at baseline, at the end of washout, and at the end of the control phase.

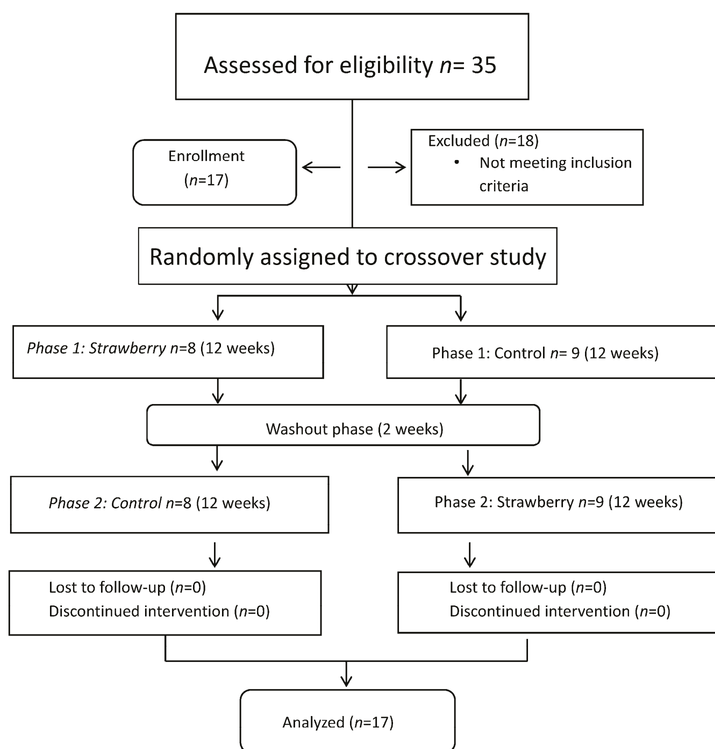


Figure 1. Study design.

Table 2. Baseline characteristics.

N	17
Age (years)	57 ± 7
Gender M/F	4/13
Anti-hypertensive medications (%)	65
Lipid lowering medications (%)	35
Oral hypoglycemic agents (%)	12
Vitamin supplement users (%)	65
Kellgren–Lawrence grade	2.1 ± 0.7

Values are in means ± SD for age (years); M: male; F: female.

We examined associations of the selected biomarkers of inflammation and cartilage degradation with knee pain scores and HAQ-DI at baseline. As shown in Table 3, in a multivariable model at baseline, IL-6 was significantly associated with constant pain, and MMP-8 with intermittent knee pain (both $p < 0.05$).

Among the selected inflammatory variables associated with knee OA measured in the study, serum IL-6 and IL-1β were significantly lower in the strawberry vs. control phase at week 12 ($p < 0.05$, Table 4), while no changes were noted in the serum hs-CRP and nitrite levels. Among the serum markers of cartilage degradation, MMP-3 was observed to be significantly lower in the strawberry vs. control phase at week 12 ($p < 0.05$, Table 4), while no significant changes were noted in MMP-8 between the two phases. As shown in Table 4, anthropometrics, blood pressure, glucose, HbA1c, lipid

profiles, and liver and kidney function tests did not differ between the strawberry and control phases of the crossover study.

Table 3. Baseline associations of knee pain scores and Health Assessment Questionnaire-Disability Index with selected markers of inflammation and cartilage degradation in obese adults with symptomatic knee osteoarthritis (linear regression coefficients) (n = 17).

Serum Variable	ICOAP (Constant Pain) %	ICOAP (Intermittent Pain) %	ICOAP (Total Pain) %	HAQ-DI
hs-CRP (mg/L)	1.9 ± 1.3	1.7 ± 1.4	1.8 ± 1.1	0.03 ± 0.04
p-value *	0.18	0.24	0.25	0.28
IL-6 (pg/mL)	5.7 ± 2.8	1.8 ± 2.9	3.6 ± 2.4	0.003 ± 0.08
p-value	**0.03**	0.23	0.37	0.42
IL-1β (pg/mL)	0.4 ± 0.3	0.04 ± 0.3	0.2 ± 0.1	0.003 ± 0.007
p-value	0.21	0.32	0.42	0.46
MMP-3 (ng/mL)	1.6 ± 1.7	1.9 ± 1.8	1.8 ± 1.5	0.03 ± 0.05
p-value	0.34	0.23	0.21	0.53
MMP-8 (ng/mL)	2.7 ± 3.4	6.9 ± 3.5	5.0 ± 2.9	0.01 ± 0.09
p-value	0.32	**0.006**	0.42	0.21

Values are estimate (beta) ± standard error (SE) obtained from a multiple linear regression model; For each serum variable, the model was adjusted for the remaining variables of inflammation and cartilage degradation in the model, as well as baseline age, body mass index (BMI), waist circumference, and energy intake as covariates; * $p < 0.05$ in bold; hs-CRP, high sensitivity C-reactive protein; IL-6: interleukin-6; IL-1β: interleukin-1β; MMP: matrix metalloproteinase; ICOAP: intermittent and constant osteoarthritis pain; HAQ-DI: health assessment questionnaire disability index.

Table 4. Anthropometrics and serum biochemical and inflammatory profiles following strawberry and control interventions in a 26-week crossover trial in obese adults with symptomatic knee osteoarthritis (n = 17/group).

Variables	Baseline	Strawberry (12-Week)	Washout (2-Week)	Control (12-Week)	*p*-Value *
BMI (kg/m^2)	39.1 ± 1.5	39.3 ± 1.4	39.3 ± 1.5	39.3 ± 1.5	0.41
Body weight (lb)	246.4 ± 7.3	245.6 ± 7.4	245.1 ± 7.1	245.0 ± 7.2	0.32
Waist circumference (inches)	46.4 ± 1.1	46.5 ± 1.1	46.1 ± 1.0	46.8 ± 1.1	0.26
Systolic blood pressure (mm Hg)	125 ± 3.0	125 ± 2.0	126 ± 3.0	127 ± 2.0	0.37
Diastolic blood pressure (mm Hg)	80 ± 2.0	82 ± 1.0	81 ± 2.0	82 ± 1.0	0.41
Fasting glucose (mg/dL)	113.4 ± 5.6	118.9 ± 8.7	114.7 ± 4.5	112.3 ± 5.1	0.31
HbA1c (%)	5.9 ± 0.2	6.1 ± 0.1	5.99 ± 0.2	6.1 ± 0.2	0.88
Total Cholesterol (mg/dL)	189 ± 6.2	188 ± 7.6	182 ± 7.0	188 ± 8.7	0.86
LDL Cholesterol (mg/dL)	109 ± 6.3	108 ± 6.9	105 ± 6.8	105 ± 7.6	0.65
HDL Cholesterol (mg/dL)	51 ± 3.0	52 ± 2.9	50 ± 2.5	54 ± 3.3	0.12
Triglycerides (mg/dL)	129 ± 14.3	136 ± 16.1	128 ± 15.0	130 ± 12.9	0.54
ALT (U/L)	40.5 ± 2.8	38.6 ± 2.3	39.5 ± 2.1	40.6 ± 2.9	0.42
AST (U/L)	30.1 ± 2.6	28.9 ± 2.3	28.6 ± 1.7	31.4 ± 2.1	0.52
Creatinine (mg/dL)	0.75 ± 0.03	0.78 ± 0.03	0.75 ± 0.03	0.77 ± 0.02	0.42
BUN (mg/dL)	15.5 ± 0.9	14.5 ± 0.7	15.3 ± 1.0	14.5 ± 1.0	0.36
hs-CRP (mg/L)	5.7 ± 1.2	4.6 ± 0.9	5.4 ± 1.1	4.8 ± 0.8	0.74
IL-6 (pg/mL)	8.8 ± 0.4	3.4 ± 0.5	8.1 ± 0.9	8.7 ± 1.4	**0.006**
IL-1β (pg/mL)	18.6 ± 4.0	7.5 ± 0.7	16.3 ± 3.1	16.2 ± 1.2	**<0.0001**
MMP-3 (ng/mL)	6.9 ± 0.6	5.3 ± 0.5	7.1 ± 0.6	6.8 ± 0.5	**0.004**
MMP-8 (ng/mL)	1.8 ± 0.3	2.2 ± 0.3	2.4 ± 0.2	2.1 ± 0.2	0.26
Nitrite (μM)	6.4 ± 0.7	9.9 ± 2.2	6.4 ± 0.8	7.5 ± 0.8	0.23

Values are means ± SEMs (Standard error of means) obtained from a linear mixed-effects model with time as within-subject factor and intervention group as a between-subject factor; * Strawberry 12-week vs. Control 12-week adjusted for Baseline; $p < 0.05$ in bold; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; BUN: blood urea nitrogen; hs-CRP, high sensitivity C-reactive protein; IL-6: interleukin-6; IL-1β: interleukin-1β; MMP: matrix metalloproteinase.

As shown in Table 5, the pain scores and HAQ-DI ratings were lower in the strawberry vs. control phase of the study. The knee pain scores measured as constant, intermittent, and total pain

using ICOAP surveys were significantly lower following the strawberry vs. control phase at week 12 ($p < 0.05$, Table 5). No differences were noted in the VAS pain scores. Among the surveys related to general health and disability index, the HAQ-DI ratings were again significantly lower in the strawberry vs. control phase at week 12 ($p < 0.05$, Table 5). The VAS health scores were not affected by the strawberry treatment.

Table 5. Measures of knee pain and quality of life indicators following strawberry and control interventions in a 26-week crossover trial in obese adults with symptomatic knee osteoarthritis (n = 17/group).

Variables	Baseline	Strawberry (12-Week)	Washout (2-Week)	Control (12-Week)	*p*-Value *
HAQ-DI	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	**0.026**
VAS PAIN	1.4 ± 0.2	0.8 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	0.17
VAS HEALTH	0.7 ± 0.1	1.0 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	0.13
ICOAP (Constant pain) %	31.8 ± 3.5	13.8 ± 3.6	32.1 ± 3.7	24.2 ± 4.1	**0.01**
ICOAP (Intermittent pain) %	38.5 ± 3.4	24.3 ± 4.7	34.1 ± 2.2	34.6 ± 3.0	**0.02**
ICOAP (Total pain) %	35.4 ± 3.1	19.4 ± 3.7	33.2 ± 2.2	29.9 ± 3.0	**0.007**

Values are means ± SEMs obtained from a linear mixed-effects model with time as within-subject factor and intervention group as a between-subject factor; * Strawberry 12-week vs. Control 12-week adjusted for Baseline; $p < 0.05$ in bold; ICOAP: intermittent and constant osteoarthritis pain; HAQ-DI: health assessment questionnaire disability index; VAS: visual analog scale.

The dietary data did not reveal any significant differences in the mean intake of macro- and micro-nutrients throughout the study (Table 6). No crossover effects were detected on any of the outcome variables.

Table 6. Dietary nutrient intakes in a 26-week crossover trial in obese adults with symptomatic knee osteoarthritis (n = 17/group).

Nutrients	Baseline	Strawberry (12-Week)	Washout (2-Week)	Control (12-Week)	*p*-Value *
Calories (kcal)	2026 ± 183	2604 ± 441	2004 ± 184	1901 ± 203	0.12
Carbohydrates (g)	215 ± 19	320 ± 56	216 ± 20	228 ± 29	0.21
Fats (g)	90 ± 12	100 ± 22	89 ± 12	78 ± 9	0.31
Proteins (g)	92 ± 11	107 ± 19	96 ± 10	76 ± 7	0.43
Saturated fats (g)	30 ± 4	32 ± 6	28 ± 3	26 ± 3	0.31
MUFA (g)	17 ± 4	22 ± 6	17 ± 4	13 ± 3	0.18
PUFA (g)	8 ± 2	12 ± 3	7 ± 1.5	6 ± 2	0.11
Fiber (g)	18 ± 2	23 ± 3	19 ± 2	20 ± 3	0.43
Vitamin C (mg)	45 ± 9	81 ± 17	42 ± 9	75 ± 28	0.77
Vitamin E (mg)	5 ± 2	7 ± 2	4 ± 1	5 ± 2	0.34
Beta-carotene (µg)	590 ± 303	890 ± 203	580 ± 311	828 ± 400	0.63

Values are means ± SEMs obtained from a linear mixed-effects model with time as within-subject factor and intervention group as a between-subject factor; * Strawberry 12-week vs. Control 12-week adjusted for Baseline; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

4. Discussion

To our knowledge, this is the first clinical study on the effects of dietary berries as a nutritional supplement on pain scores and key biomarkers of inflammation in obese adults with radiographic evidence of knee OA. Using a multi questionnaire approach, strawberry supplementation led to significant decreases in constant, intermittent, and total knee pain scores, and an improved disability index and overall health scores. Serum biomarkers of inflammation and cartilage degradation that have been associated with pain and dysfunction in knee OA, especially IL-6, IL-1β, and MMP-3, were also shown to be significantly lower in the strawberry-supplemented group. These findings support a role for foods high in bioactive compounds, such as strawberries, as an alternative or complementary

treatment option in pain management that may also reduce surrogate markers of disease progression in knee OA.

Pain relief is one of the major targets of OA management. Symptoms of pain in OA are associated with inflammation and oxidative stress, cartilage degradation, and joint space narrowing [32–34]. It is therefore logical to propose that antioxidant supplements may be of benefit. Strawberries are naturally rich in antioxidant polyphenols, and thus were selected for our study [35]. Participants rated their pain in the range of mild to moderate intensity at baseline using the VAS as well as the ICOAP questionnaires. Other polyphenol containing supplements, such as curcumin [13,36], green tea [16], and herbal tea supplements [17], have also shown significant decreases in knee pain scores in participants with similar intensity of knee pain as in our study. However, none of these studies reported effects on systemic markers of inflammation and disease progression underlying knee OA.

Together with previous reports, our findings support the analgesic effects of dietary polyphenols in adults with mild to moderate knee pain. Pain measurement in OA has been largely determined by questionnaires, such as those based on quality of life indicators. Meanwhile, physical examination and radiography have been used to stage the disease. We used the VAS, HAQ-DI, and ICOAP questionnaires: these have been widely employed to assess knee pain, quality of life, and disability in adults with OA [29,30]. The ICOAP questionnaire is endorsed by the Osteoarthritis Research Society International (OARSI). It has been validated in large multi-country studies, and correlates well with other commonly used methods, such as the Western Ontario and McMaster Universities' Osteoarthritis Index (WOMAC) scores [28,37]. Based on our study findings, strawberries consistently improved pain scores as observed across all three sub-scales of ICOAP, evaluating constant, intermittent, and total pain; they also lowered HAQ-DI scores, reflecting functional improvement. We did not observe any difference in pain scores assessed by VAS survey in our participants. These differences could be explained by the visual expression of general pain intensity used in VAS scoring, when compared to the OA-specific magnitude of knee pain numerically rated by the ICOAP questionnaires. Based on the strengths and limitations of each assessment tool for pain, it is generally recommended to administer more than one questionnaire to capture the multi-dimensional aspects of adult pain. These findings merit follow up in larger trials to validate the findings.

Inflammation is believed to play a pivotal role in the pathophysiology of OA. Multiple cytokines and inflammatory molecules, especially CRP, IL-6, and IL-1β, and free radicals, such as nitric oxide, are implicated in the progression of OA [38,39]. Inflamed chondrocytes then produce MMPs, leading to cartilage degradation and progression of OA [40]. In experimental models of OA, green tea, curcumin, and some herbal supplements may reduce inflammatory molecules and MMPs [41,42], while few of the reported clinical studies have determined the effects of the dietary bioactive compounds on inflammatory mediators. In a 16-week study assessing the effects of a high-polyphenol rosmarinic acid tea on pain in participants with knee OA, serum CRP was reported only at baseline but not after the intervention [17]. Among the studies showing improvements in knee pain in OA following curcumin supplementation [13,43,44], only one study reported data on inflammatory markers, including IL-6; however, these did not differ between the intervention and placebo groups after six weeks' treatment [43]. Thus, the study of inflammatory mediators is limited in previous reports of dietary supplements for OA.

In our study, 12 weeks of strawberry supplementation resulted in a significant decrease in IL-6, IL-1β, and MMP-3 in obese participants with knee OA, consistent with anti-inflammatory effects of dietary berries in OA management. These clinical observations are consistent with data showing that blueberry and raspberry extracts lower pain, inflammation, and edema, and articular destruction in experimental arthritis [21,22]. Metalloproteinases zinc-dependent enzymes (MMPs) play a key role in extracellular matrix remodeling and cartilage metabolism in knee OA. Among the various isoforms of MMPs, MMP-3 plays an important role in cartilage degradation, and has been shown to be responsive to therapeutic agents in patients with various stages of OA [12,45,46]. On the other hand, MMP-8 has been implicated in the degradation of already compromised cartilage matrix, and a few clinical

studies have examined its response to therapeutic agents, and revealed conflicting results [45,47,48]. Thus, future studies must address the role of dietary polyphenolic compounds on a comprehensive panel of serum MMPs to identify clinically responsive biomarkers in OA management.

Obesity has been strongly correlated with knee OA, and consequently weight loss studies, especially the Intensive Diet and Exercise for Arthritis (IDEA) trial, have demonstrated significant decreases in IL-6 and improvements in knee pain and function following diet and exercise interventions in obese older adults [31]. Interestingly, the magnitude of the decrease in serum IL-6 in our study following the 12-week strawberry intervention was much larger than what was noted in the IDEA trial following an 18-month lifestyle intervention [31]. IL-6 is a key inflammatory molecule that accelerates articular degradation and OA progression, and higher levels of systemic IL-6 are a significant predictor of OA [49]. Furthermore, a reduction of IL-6 levels can significantly improve the metabolic syndrome, also considered a risk factor of OA [50]. The IDEA trial also reported a concomitant decrease in CRP in obese older adults undergoing 5% total weight and fat mass loss following a dietary and exercise intervention [51]. However, CRP was not significantly altered in the present study, and it may be that weight loss is essential to affect this marker of inflammation. Future studies must assess the combined effects of antioxidant bioactive compounds with weight loss in improving inflammatory profiles in knee OA.

Our study has limitations that affect the interpretation and generalizability of our findings. These include a small sample size, the absence of a dose–response design to assess effects at low vs. high dose of strawberries, and the absence of a non-OA control group. Participants had mild-to-moderate radiographic knee OA at baseline (Kellgren–Lawrence scores of 2.1) and mild-to-moderate knee pain. Whether patients with more severe knee OA (Kellgren–Lawrence score of 4) and higher levels of pain would benefit from strawberry intervention needs further investigation. We did not measure other biomarkers of OA pathology, such as those related to oxidative damage, or simultaneously measure these biomarkers in synovial fluid that would provide a more accurate determination of changes specific to the knee. Also, we did not assess the radiological outcomes at the end of the intervention. Also, being conducted in obese participants with mild-to-moderate symptoms of knee OA, our study findings may not be generalizable to the non-obese population or OA caused by sports injuries and other trauma, or to those needing pain relief after knee surgery.

The strengths of our study include a randomized, controlled cross-over study design, which accounts for most of the inter-individual variations in parallel arm studies. Also, based on the administration of a control powder that matched the freeze-dried strawberries in sensory qualities, we were able to keep the participants and study coordinators blinded to the identity of the test agents. In addition, we excluded participants who were taking supplements, such as fish oil and other herbal supplements for pain relief, as well as those participating in a weight loss program, and thus were able to exclude potential confounding by these factors.

5. Conclusions

In conclusion, our pilot study provides evidence on the role of strawberry bioactive compounds, as a rich source of polyphenols and nutrients, in improving pain and inflammation in obese adults with mild-to-moderate knee OA when compared to a control group. Given the economic burden of obesity and related conditions, including knee OA, our study suggests that simple dietary intervention, i.e., the addition of berries, may have a significant impact on pain, inflammation, and overall quality of life in obese adults with OA.

Acknowledgments: This study was made possible by the strawberry and control powder generously provided by the California Strawberry Commission (Watsonville, CA, USA). Support for this study was obtained from the Jim and Lynne Professorship Endowments at OSU and the NIH grant U54GM104938 (Oklahoma Shared Clinical and Translational Resource), OUHSC. The authors also wish to thank Judith James and Eliza Chakravarty in the initial project planning. In addition, the authors thank all OSU and OUHSC faculty and staff who participated as volunteers and made this study possible, as well as Janice Gales for assistance in recruitment and participant follow ups.

Author Contributions: J.S., R.H.S., J.R.B., B.T.K., N.B. and A.B. designed the study, performed the participant recruitment and the laboratory analyses, and wrote, reviewed, and edited the manuscript. Y.D.Z. performed the statistical analyses. T.J.L. assisted with data interpretation and the writing of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no competing interests.

References

1. Hochberg, M.C.; Altman, R.D.; April, K.T.; Benkhalti, M.; Guyatt, G.; McGowan, J.; Towheed, T.; Welch, V.; Wells, G.; Tugwell, P.; et al. American College of Rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. *Arthritis Care Res. (Hoboken)* **2012**, *64*, 465–474. [CrossRef] [PubMed]
2. Meneses, S.R.; Goode, A.P.; Nelson, A.E.; Lin, J.; Jordan, J.M.; Allen, K.D.; Bennell, K.L.; Lohmander, L.S.; Fernandes, L.; Hochberg, M.C.; et al. Clinical algorithms to aid osteoarthritis guideline dissemination. *Osteoarthr. Cartil.* **2016**. [CrossRef]
3. McAlindon, T.E.; Bannuru, R.R.; Sullivan, M.C.; Arden, N.K.; Berenbaum, F.; Bierma-Zeinstra, S.M.; Hawker, G.A.; Henrotin, Y.; Hunter, D.J.; Kawaguchi, H.; et al. OARSI guidelines for the non-surgical management of knee osteoarthritis. *Osteoarthr. Cartil.* **2014**, *22*, 363–388. [CrossRef] [PubMed]
4. Wandel, S.; Juni, P.; Tendal, B.; Nuesch, E.; Villiger, P.M.; Welton, N.J.; Reichenbach, S.; Trelle, S. Effects of glucosamine, chondroitin, or placebo in patients with osteoarthritis of hip or knee: Network meta-analysis. *BMJ* **2010**, *341*, c4675. [CrossRef] [PubMed]
5. Christiansen, B.A.; Bhatti, S.; Goudarzi, R.; Emami, S. Management of Osteoarthritis with Avocado/Soybean Unsaponifiables. *Cartilage* **2015**, *6*, 30–44. [CrossRef] [PubMed]
6. Ameye, L.G.; Chee, W.S. Osteoarthritis and nutrition. From nutraceuticals to functional foods: A systematic review of the scientific evidence. *Arthr. Res. Ther.* **2006**, *8*, R127. [CrossRef] [PubMed]
7. Grotle, M.; Hagen, K.B.; Natvig, B.; Dahl, F.A.; Kvien, T.K. Obesity and osteoarthritis in knee, hip and/or hand: An epidemiological study in the general population with 10 years follow-up. *BMC Musculoskelet. Disord.* **2008**, *9*, 132. [CrossRef] [PubMed]
8. Thijssen, E.; van Caam, A.; van der Kraan, P.M. Obesity and osteoarthritis, more than just wear and tear: Pivotal roles for inflamed adipose tissue and dyslipidaemia in obesity-induced osteoarthritis. *Rheumatology (Oxf.)* **2015**, *54*, 588–600. [CrossRef] [PubMed]
9. Perruccio, A.V.; Chandran, V.; Power, J.D.; Kapoor, M.; Mahomed, N.N.; Gandhi, R. Systemic inflammation and painful joint burden in osteoarthritis: A matter of sex? *Osteoarthr. Cartil.* **2017**, *25*, 53–59. [CrossRef] [PubMed]
10. Larsson, S.; Englund, M.; Struglics, A.; Lohmander, L.S. Interleukin-6 and tumor necrosis factor alpha in synovial fluid are associated with progression of radiographic knee osteoarthritis in subjects with previous meniscectomy. *Osteoarthr. Cartil.* **2015**, *23*, 1906–1914. [CrossRef] [PubMed]
11. Ling, S.M.; Patel, D.D.; Garnero, P.; Zhan, M.; Vaduganathan, M.; Muller, D.; Taub, D.; Bathon, J.M.; Hochberg, M.; Abernethy, D.R.; et al. Serum protein signatures detect early radiographic osteoarthritis. *Osteoarthr. Cartil.* **2009**, *17*, 43–48. [CrossRef] [PubMed]
12. Pelletier, J.P.; Raynauld, J.P.; Caron, J.; Mineau, F.; Abram, F.; Dorais, M.; Haraoui, B.; Choquette, D.; Martel-Pelletier, J. Decrease in serum level of matrix metalloproteinases is predictive of the disease-modifying effect of osteoarthritis drugs assessed by quantitative MRI in patients with knee osteoarthritis. *Ann. Rheum. Dis.* **2010**, *69*, 2095–2101. [CrossRef] [PubMed]
13. Panahi, Y.; Rahimnia, A.R.; Sharafi, M.; Alishiri, G.; Saburi, A.; Sahebkar, A. Curcuminoid treatment for knee osteoarthritis: A randomized double-blind placebo-controlled trial. *Phytother. Res.* **2014**, *28*, 1625–1631. [CrossRef] [PubMed]
14. Panahi, Y.; Alishiri, G.H.; Parvin, S.; Sahebkar, A. Mitigation of Systemic Oxidative Stress by Curcuminoids in Osteoarthritis: Results of a Randomized Controlled Trial. *J. Diet. Suppl.* **2016**, *13*, 209–220. [CrossRef] [PubMed]

15. Naderi, Z.; Mozaffari-Khosravi, H.; Dehghan, A.; Nadjarzadeh, A.; Huseini, H.F. Effect of ginger powder supplementation on nitric oxide and C-reactive protein in elderly knee osteoarthritis patients: A 12-week double-blind randomized placebo-controlled clinical trial. *J. Tradit. Complement. Med.* **2016**, *6*, 199–203. [CrossRef] [PubMed]
16. Hashempur, M.H.; Sadrneshin, S.; Mosavat, S.H.; Ashraf, A. Green tea (*Camellia sinensis*) for patients with knee osteoarthritis: A randomized open-label active-controlled clinical trial. *Clin. Nutr.* **2016**. [CrossRef] [PubMed]
17. Connelly, A.E.; Tucker, A.J.; Tulk, H.; Catapang, M.; Chapman, L.; Sheikh, N.; Yurchenko, S.; Fletcher, R.; Kott, L.S.; Duncan, A.M.; et al. High-rosmarinic acid spearmint tea in the management of knee osteoarthritis symptoms. *J. Med. Food* **2014**, *17*, 1361–1367. [CrossRef] [PubMed]
18. Cassidy, A.; O'Reilly, E.J.; Kay, C.; Sampson, L.; Franz, M.; Forman, J.P.; Curhan, G.; Rimm, E.B. Habitual intake of flavonoid subclasses and incident hypertension in adults. *Am. J. Clin. Nutr.* **2011**, *93*, 338–347. [CrossRef] [PubMed]
19. Wedick, N.M.; Pan, A.; Cassidy, A.; Rimm, E.B.; Sampson, L.; Rosner, B.; Willett, W.; Hu, F.B.; Sun, Q.; van Dam, R.M. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. *Am. J. Clin. Nutr.* **2012**, *95*, 925–933. [CrossRef] [PubMed]
20. Cassidy, A.; Rogers, G.; Peterson, J.J.; Dwyer, J.T.; Lin, H.; Jacques, P.F. Higher dietary anthocyanin and flavonol intakes are associated with anti-inflammatory effects in a population of US adults. *Am. J. Clin. Nutr.* **2015**, *102*, 172–181. [CrossRef] [PubMed]
21. Figueira, M.E.; Camara, M.B.; Direito, R.; Rocha, J.; Serra, A.T.; Duarte, C.M.; Fernandes, A.; Freitas, M.; Fernandes, E.; Marques, M.C.; et al. Chemical characterization of a red raspberry fruit extract and evaluation of its pharmacological effects in experimental models of acute inflammation and collagen-induced arthritis. *Food Funct.* **2014**, *5*, 3241–3251. [CrossRef] [PubMed]
22. Figueira, M.E.; Oliveira, M.; Direito, R.; Rocha, J.; Alves, P.; Serra, A.T.; Duarte, C.; Bronze, R.; Fernandes, A.; Brites, D.; et al. Protective effects of a blueberry extract in acute inflammation and collagen-induced arthritis in the rat. *Biomed. Pharmacother.* **2016**, *83*, 1191–1202. [CrossRef] [PubMed]
23. Basu, A.; Du, M.; Leyva, M.J.; Sanchez, K.; Betts, N.M.; Wu, M.; Aston, C.E.; Lyons, T.J. Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *J. Nutr.* **2010**, *140*, 1582–1587. [CrossRef]
24. Basu, A.; Betts, N.M.; Nguyen, A.; Newman, E.D.; Fu, D.; Lyons, T.J. Freeze-dried strawberries lower serum cholesterol and lipid peroxidation in adults with abdominal adiposity and elevated serum lipids. *J. Nutr.* **2014**, *144*, 830–837. [CrossRef] [PubMed]
25. Basu, A.; Wilkinson, M.; Penugonda, K.; Simmons, B.; Betts, N.M.; Lyons, T.J. Freeze-dried strawberry powder improves lipid profile and lipid peroxidation in women with metabolic syndrome: Baseline and post intervention effects. *Nutr. J.* **2009**, *8*, 43. [CrossRef] [PubMed]
26. Peat, G.; Thomas, E.; Duncan, R.; Wood, L.; Hay, E.; Croft, P. Clinical classification criteria for knee osteoarthritis: Performance in the general population and primary care. *Ann. Rheum. Dis.* **2006**, *65*, 1363–1367. [CrossRef] [PubMed]
27. Seeram, N.P.; Lee, R.; Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* **2004**, *348*, 63–68. [CrossRef] [PubMed]
28. Mehta, S.P.; Sankar, A.; Venkataramanan, V.; Lohmander, L.S.; Katz, J.N.; Hawker, G.A.; Gossec, L.; Roos, E.M.; Maillefert, J.F.; Kloppenburg, M.; et al. Cross-cultural validation of the ICOAP and physical function short forms of the HOOS and KOOS in a multi-country study of patients with hip and knee osteoarthritis. *Osteoarthr. Cartil.* **2016**, *24*, 2077–2081. [CrossRef]
29. Risser, R.C.; Hochberg, M.C.; Gaynor, P.J.; D'Souza, D.N.; Frakes, E.P. Responsiveness of the Intermittent and Constant Osteoarthritis Pain (ICOAP) scale in a trial of duloxetine for treatment of osteoarthritis knee pain. *Osteoarthr. Cartil.* **2013**, *21*, 691–694. [CrossRef] [PubMed]
30. Kim, H.L.; Kim, D.; Jang, E.J.; Lee, M.Y.; Song, H.J.; Park, S.Y.; Cha, H.S.; Choe, J.Y.; Chung, W.T.; Hong, S.J.; et al. Mapping health assessment questionnaire disability index (HAQ-DI) score, pain visual analog scale (VAS), and disease activity score in 28 joints (DAS28) onto the EuroQol-5D (EQ-5D) utility score with the KORean Observational study Network for Arthritis (KORONA) registry data. *Rheumatol. Int.* **2016**, *36*, 505–513.

31. Messier, S.P.; Mihalko, S.L.; Legault, C.; Miller, G.D.; Nicklas, B.J.; DeVita, P.; Beavers, D.P.; Hunter, D.J.; Lyles, M.F.; Eckstein, F.; et al. Effects of intensive diet and exercise on knee joint loads, inflammation, and clinical outcomes among overweight and obese adults with knee osteoarthritis: The IDEA randomized clinical trial. *JAMA* **2013**, *310*, 1263–1273. [CrossRef] [PubMed]
32. Andriacchi, T.P.; Favre, J. The nature of in vivo mechanical signals that influence cartilage health and progression to knee osteoarthritis. *Curr. Rheumatol. Rep.* **2014**, *16*, 463. [CrossRef]
33. Muraki, S.; Akune, T.; En-Yo, Y.; Yoshida, M.; Suzuki, T.; Yoshida, H.; Ishibashi, H.; Tokimura, F.; Yamamoto, S.; Tanaka, S.; et al. Joint space narrowing, body mass index, and knee pain: The ROAD study (OAC1839R1). *Osteoarthr. Cartil.* **2015**, *23*, 874–881. [CrossRef] [PubMed]
34. Regan, E.; Flannelly, J.; Bowler, R.; Tran, K.; Nicks, M.; Carbone, B.D.; Glueck, D.; Heijnen, H.; Mason, R.; Crapo, J.; et al. Extracellular superoxide dismutase and oxidant damage in osteoarthritis. *Arthr. Rheum.* **2005**, *52*, 3479–3491. [CrossRef] [PubMed]
35. Basu, A.; Nguyen, A.; Betts, N.M.; Lyons, T.J. Strawberry as a functional food: An evidence-based review. *Crit. Rev. Food Sci. Nutr.* **2014**, *54*, 790–806. [CrossRef] [PubMed]
36. Belcaro, G.; Dugall, M.; Luzzi, R.; Ledda, A.; Pellegrini, L.; Cesarone, M.R.; Hosoi, M.; Errichi, M. Meriva(R) + Glucosamine versus Condroitin + Glucosamine in patients with knee osteoarthritis: An observational study. *Eur. Rev. Med. Pharmacol. Sci.* **2014**, *18*, 3959–6393. [PubMed]
37. Ruyssen-Witrand, A.; Fernandez-Lopez, C.J.; Gossec, L.; Anract, P.; Courpied, J.P.; Dougados, M. Psychometric properties of the OARSI/OMERACT osteoarthritis pain and functional impairment scales: ICOAP, KOOS-PS and HOOS-PS. *Clin. Exp. Rheumatol.* **2011**, *29*, 231–237. [PubMed]
38. Mabey, T.; Honsawek, S.; Saetan, N.; Poovorawan, Y.; Tanavalee, A.; Yuktanandana, P. Angiogenic cytokine expression profiles in plasma and synovial fluid of primary knee osteoarthritis. *Int. Orthop.* **2014**, *38*, 1885–1892. [CrossRef] [PubMed]
39. Kapoor, M.; Martel-Pelletier, J.; Lajeunesse, D.; Pelletier, J.P.; Fahmi, H. Role of proinflammatory cytokines in the pathophysiology of osteoarthritis. *Nat. Rev. Rheumatol.* **2011**, *7*, 33–42. [CrossRef]
40. Burrage, P.S.; Mix, K.S.; Brinckerhoff, C.E. Matrix metalloproteinases: Role in arthritis. *Front. Biosci.* **2006**, *11*, 529–543. [CrossRef] [PubMed]
41. Comblain, F.; Sanchez, C.; Lesponne, I.; Balligand, M.; Serisier, S.; Henrotin, Y. Curcuminoids extract, hydrolyzed collagen and green tea extract synergically inhibit inflammatory and catabolic mediator's synthesis by normal bovine and osteoarthritic human chondrocytes in monolayer. *PLoS ONE* **2015**, *10*, e0121654. [CrossRef] [PubMed]
42. Nirmal, P.S.; Jagtap, S.D.; Narkhede, A.N.; Nagarkar, B.E.; Harsulkar, A.M. New herbal composition (OA-F2) protects cartilage degeneration in a rat model of collagenase induced osteoarthritis. *BMC Complement. Altern. Med.* **2017**, *17*, 6. [CrossRef] [PubMed]
43. Rahimnia, A.R.; Panahi, Y.; Alishiri, G.; Sharafi, M.; Sahebkar, A. Impact of Supplementation with Curcuminoids on Systemic Inflammation in Patients with Knee Osteoarthritis: Findings from a Randomized Double-Blind Placebo-Controlled Trial. *Drug. Res. (Stuttg.)* **2015**, *65*, 521–525. [CrossRef] [PubMed]
44. Nakagawa, Y.; Mukai, S.; Yamada, S.; Matsuoka, M.; Tarumi, E.; Hashimoto, T.; Tamura, C.; Imaizumi, A.; Nishihira, J.; Nakamura, T.; et al. Short-term effects of highly-bioavailable curcumin for treating knee osteoarthritis: A randomized, double-blind, placebo-controlled prospective study. *J. Orthop. Sci.* **2014**, *19*, 933–939. [CrossRef]
45. Manicourt, D.H.; Azria, M.; Mindeholm, L.; Thonar, E.J.; Devogelaer, J.P. Oral salmon calcitonin reduces Lequesne's algofunctional index scores and decreases urinary and serum levels of biomarkers of joint metabolism in knee osteoarthritis. *Arthr. Rheum.* **2006**, *54*, 3205–3211. [CrossRef] [PubMed]
46. Martel-Pelletier, J.; Raynauld, J.P.; Mineau, F.; Abram, F.; Paiement, P.; Delorme, P.; Pelletier, J. Levels of serum biomarkers from a two-year multicentre trial are associated with treatment response on knee osteoarthritis cartilage loss as assessed by magnetic resonance imaging: An exploratory study. *Arthr. Res. Ther.* **2017**, *19*, 169. [CrossRef] [PubMed]
47. Kullich, W.C.; Niksic, F.; Klein, G. Effect of nimesulide on metalloproteinases and matrix degradation in osteoarthritis: A pilot clinical study. *Int. J. Clin. Pract. Suppl.* **2002**, *128*, 24–29.
48. Bellometti, S.; Richelmi, P.; Tassoni, T.; Berte, F. Production of matrix metalloproteinases and their inhibitors in osteoarthritic patients undergoing mud bath therapy. *Int. J. Clin. Pharmacol. Res.* **2005**, *25*, 77–94. [PubMed]

49. Livshits, G.; Zhai, G.; Hart, D.J.; Kato, B.S.; Wang, H.; Williams, F.M.; Spector, T.D. Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: The Chingford Study. *Arthr. Rheum.* **2009**, *60*, 2037–2045. [CrossRef] [PubMed]
50. Esposito, K.; Marfella, R.; Ciotola, M.; Di Palo, C.; Giugliano, F.; Giugliano, G.; D'Armiento, M.; D'Andrea, F.; Giugliano, D. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: A randomized trial. *JAMA* **2004**, *292*, 1440–1446. [CrossRef] [PubMed]
51. Beavers, K.M.; Beavers, D.P.; Newman, J.J.; Anderson, A.M.; Loeser, R.F., Jr.; Nicklas, B.J.; Lyles, M.F.; Miller, G.D.; Mihalko, S.L.; Messier, S.P. Effects of total and regional fat loss on plasma CRP and IL-6 in overweight and obese, older adults with knee osteoarthritis. *Osteoarthr. Cartil.* **2015**, *23*, 249–256. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Coffee Consumption and Risk of Biliary Tract Cancers and Liver Cancer: A Dose–Response Meta-Analysis of Prospective Cohort Studies

Justyna Godos [1,*], Agnieszka Micek [2], Marina Marranzano [1], Federico Salomone [3], Daniele Del Rio [4,5] and Sumantra Ray [5,6]

1 Department of Medical and Surgical Sciences and Advanced Technologies "G.F. Ingrassia", University of Catania, 95124 Catania, Italy; marranz@unict.it
2 Department of Epidemiology and Population Studies, Jagiellonian University Medical College, 31-008 Krakow, Poland; agnieszka.micek@uj.edu.pl
3 Division of Gastroenterology, Ospedale di Acireale, Azienda Sanitaria Provinciale di Catania, 95024 Catania, Italy; federicosalomone@rocketmail.com
4 Department of Food and Drugs, University of Parma, 43121 Parma, Italy; daniele.delrio@unipr.it
5 NNEdPro Global Centre for Nutrition and Health (Affiliated with: Cambridge University Health Partners and the British Dietetic Association), St John's Innovation Centre, Cambridge CB4 0WS, UK; sumantra.ray@mrc-ewl-hnr.cam.ac.uk
6 Medical Research Council (MRC) Human Nutrition Research Unit, Cambridge CB1 9NL, UK
* Correspondence: justyna.godos@student.uj.edu.pl; Tel.: +39-095-378-2180

Received: 20 July 2017; Accepted: 24 August 2017; Published: 28 August 2017

Abstract: Background: A meta-analysis was conducted to summarize the evidence from prospective cohort and case-control studies regarding the association between coffee intake and biliary tract cancer (BTC) and liver cancer risk. Methods: Eligible studies were identified by searches of PubMed and EMBASE databases from the earliest available online indexing year to March 2017. The dose–response relationship was assessed by a restricted cubic spline model and multivariate random-effect meta-regression. A stratified and subgroup analysis by smoking status and hepatitis was performed to identify potential confounding factors. Results: We identified five studies on BTC risk and 13 on liver cancer risk eligible for meta-analysis. A linear dose–response meta-analysis did not show a significant association between coffee consumption and BTC risk. However, there was evidence of inverse correlation between coffee consumption and liver cancer risk. The association was consistent throughout the various potential confounding factors explored including smoking status, hepatitis, etc. Increasing coffee consumption by one cup per day was associated with a 15% reduction in liver cancer risk (RR 0.85; 95% CI 0.82 to 0.88). Conclusions: The findings suggest that increased coffee consumption is associated with decreased risk of liver cancer, but not BTC.

Keywords: coffee; caffeine; gallbladder cancer; biliary tract cancer; liver cancer; hepatitis; meta-analysis; dose–response

1. Introduction

Coffee is one of the most consumed beverages worldwide and it has been associated with a number of benefits on human health including a decreased risk of all-cause, cardiovascular, and cancer mortality [1–3]. Coffee is composed of a variety of compounds, some of which have been reported to have an impact on liver health [4]. Caffeine, a major component in coffee has been proposed to exert anti-carcinogenic effects toward up-regulation of antioxidant-responsive element (ARE)-mediated signalling [5], while phenolic compounds in coffee have been shown to exert anti-oxidant and anti-inflammatory effects [6]. In addition, coffee diterpenes have potential anti-carcinogenic effects [6].

However, besides the contribution of individual compounds or groups of compounds to liver health, synergistic effects are also possible.

Gallbladder (GB) cancer is a highly fatal malignancy with notable geographical variations and a higher incidence in women. The aetiology of biliary tract cancer (BTC), including GB cancer and bile duct cancer (BDC) is poorly understood. One of the main risk factors has been hypothesized to be prolonged exposure to gallstones associated with chronic inflammation [7], which may increase the risk of BTC [8]. It has also been suggested that dietary factors contributing to gallstone formation can play a role in aetiology of BTC. Caffeinated and decaffeinated coffee consumption stimulates cholecystokinin release, which in turn stimulates the smooth muscle of gallbladder, causing its contraction [9]. However, gallbladder contraction may cause pain/colics in patients with gallstones. Moreover, coffee intake decreases cholesterol crystallization in bile, preventing gallstone formation [10] and modulating inflammation associated with the presence of gallstones [7].

Among the main targets of the healthful effects of coffee, the liver in particular, appears to benefit from coffee consumption due to the improvement of lipid metabolism regulation and decreased rates of liver steatosis and non-alcoholic steatohepatitis, which in turn may decrease the risk of consequent inflammation and fibrosis [11]. A recent systematic review showed that coffee consumption was consistently associated with lower rates of chronic liver disease and cirrhosis [12]. Interestingly, several studies have examined the impact of coffee consumption on fibrosis severity in patients with chronic viral hepatitis and have shown decreased rates of liver disease progression in coffee drinkers [13]. Recently, meta-analyses on coffee consumption and liver cancer risk have been published [14,15], but they lacked data on BTC risk, dose–response analyses, or analysis of possible confounding factors. The aim of the present study was to perform a meta-analysis of observational studies on coffee consumption and liver and BTC cancers, including the dose–response relation while taking into account the role of several potential confounding factors.

2. Materials and Methods

Meta-Analysis of Observational Studies in Epidemiology (MOOSE) protocols were followed throughout design, execution, analysis and reporting of this meta-analysis (Table S1) [16].

2.1. Search Strategy

We conducted a comprehensive literature search using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and EMBASE (http://www.embase.com/) databases from the earliest available online indexing year to March 2017, with English-language restriction. Search terms included the following: (coffee OR caffeine OR beverages) and (extrahepatic OR gallbladder OR biliary tract OR liver OR hepatocellular) and (cancer OR carcinoma OR neoplasm) (Table S2). Two authors separately screened and retrieved the studies. We included prospective and case-control studies that evaluated association between dietary coffee intake and risk of extrahepatic/hepatic cancer in generally healthy adults. Studies were included if they provided corresponding risk estimates such as RRs (Risk Ratios), HRs (Hazard Ratios), or ORs (Odds Ratios). We excluded studies that reported insufficient statistics or insufficient coffee consumption categories (less than three; Table S3). Reference lists of included manuscripts were also examined for additional studies not previously identified. When duplicate publications from the same study were identified, we included the report that provided the largest number of cases/entire cohort or with the longest follow-up for each endpoint of interest. Full-texts of potentially relevant articles were assessed independently for eligibility by two authors.

2.2. Data Extraction

Data were abstracted from each identified study using a standardized extraction form. The following information was collected: (1) first author name; (2) year of publication; (3) study cohort name; (4) country; (5) number of participants; (6) sex of participants; (7) age range of the study population at baseline; (8) categories of consumption; (9) follow-up period; (10) endpoints and cases;

(11) distributions of cases and person-years, HRs, and 95% CIs for all categories of exposure; and (12) covariates used in adjustments. This process was performed independently by two authors and discrepancies were discussed and resolved by consensus. The quality of included studies was assessed according to the Newcastle–Ottawa Quality Assessment Scale [17], which consists of three variables of quality as follows, selection (4 points), comparability (2 points), and outcome (3 points), for a total score of 9 points (9 representing the highest quality).

2.3. Statistical Analysis

In this meta-analysis, ORs and HRs were deemed equivalent to relative risks (RRs) [18]. ORs, RRs and HRs with 95% CI for all categories of exposure were extracted for the analysis and random-effects models were used to calculate pooled RR with 95% CI for the highest versus lowest category of exposure. The highest versus lowest analysis was performed to determine the relationship between coffee intake and risk of BTC and liver cancer. We included gallbladder cancer and extrahepatic/intrahepatic bile duct cancer in the same analysis, as their aetiology is similar [7]. The risk estimate from the most fully adjusted models in the analysis of the pooled RR was used. Heterogeneity was assessed using the Q test and I^2 statistic. The level of significance equal to 0.10 was used for the Q test. The I^2 statistic represented the amount of total variation that could be attributed to heterogeneity. I^2 values ≤25%, 25–50%, 50–75%, and >75% indicated no, small, moderate, and significant heterogeneity, respectively. A sensitivity analysis by exclusion of one study at a time was performed to evaluate the stability of results and potential sources of heterogeneity. Subgroup analysis was only performed for liver cancer risk, in order to check for potential source of heterogeneity according to study design, gender and geographical area. To test for potential confounders/effect modifiers, subgroup analyses were performed according to smoking status, coffee type and hepatitis. Publication bias was evaluated by a visual investigation of funnel plots for potential asymmetry.

A dose–response analysis was performed using the method of Greenland and Longnecker to calculate study-specific liner and non-linear trend (generalized least-squares, GLS) based on results across categories of coffee intake [19,20]. Data were extracted on the level of coffee intake, distributions of cases and person-years (when available), and ORs/RRs/HRs with 95% CIs for ≥3 exposure categories. The median or mean intake of coffee in each category was assigned to the corresponding OR/RR/HR with the 95% CI for each study. When coffee consumption was reported in a range of intake, the midpoint of the range was used. When the highest category was open ended, we assumed the width of the category to be the same as the adjacent category. When the lowest category was open ended, we set the lower boundary to zero. Two-stage random-effects dose–response meta-analysis was performed to examine linear and non-linear relationship between coffee intake and risk of biliary tract cancer and liver cancer. In the first stage the method of Greenland and Longnecker (generalized least-squares, GLS) was used to calculate study-specific coefficients on the basis of results across categories of coffee intake taking into account the correlation within each set of retrieved ORs/RRs/HRs [19,20]. Non-linear dose–response analysis was modelled using restricted cubic splines with 3 knots at fixed percentiles (25%, 50%, and 75%) of the distribution [21]. The coefficients that had been estimated within each study by performing random-effects meta-analysis were combined. In linear dose–response meta-analysis the method of DerSimonian and Laird was used and in non-linear dose–response meta-analysis the multivariate extension of the method of moments was used to estimate the relative risks. We calculated an overall P-value by testing that the 2 regression coefficients were simultaneously equal to zero. We then calculated a P-value for non-linearity by testing that the coefficient of the second spline was equal to zero. All analyses were performed with R software Version 3.0.3, using dosresmeta and mvmeta packages (Development Core Team, Vienna, Austria).

3. Results

3.1. Study Characteristics

The study selection process of eligible studies is presented in Figure 1. For the analysis on the association between coffee consumption and BTC risk five studies were eligible [22–26], one of which was a pooling project of nine cohort studies [25], two were studies comprising three prospective cohorts [23,24], and two were case-control studies [22,26]. Eligible studies included 1,375,626 participants and 726 BTC cases. The main characteristics of the studies included in the meta-analysis are summarized in Table 1. Six studies provided data for men and women separately [25,27–31]. Four studies provided data on type of coffee consumed [25,32–34], six on smoking status [25,27,28,30,31,35], and six on hepatitis [27,29,30,33,36,37]. Three studies were conducted in USA [22,25,26], one in Europe [23] and one in Asia [24]. The follow-up in prospective cohort studies ranged from about 13 to 20 years, and the age range at study baseline was 30–84 years.

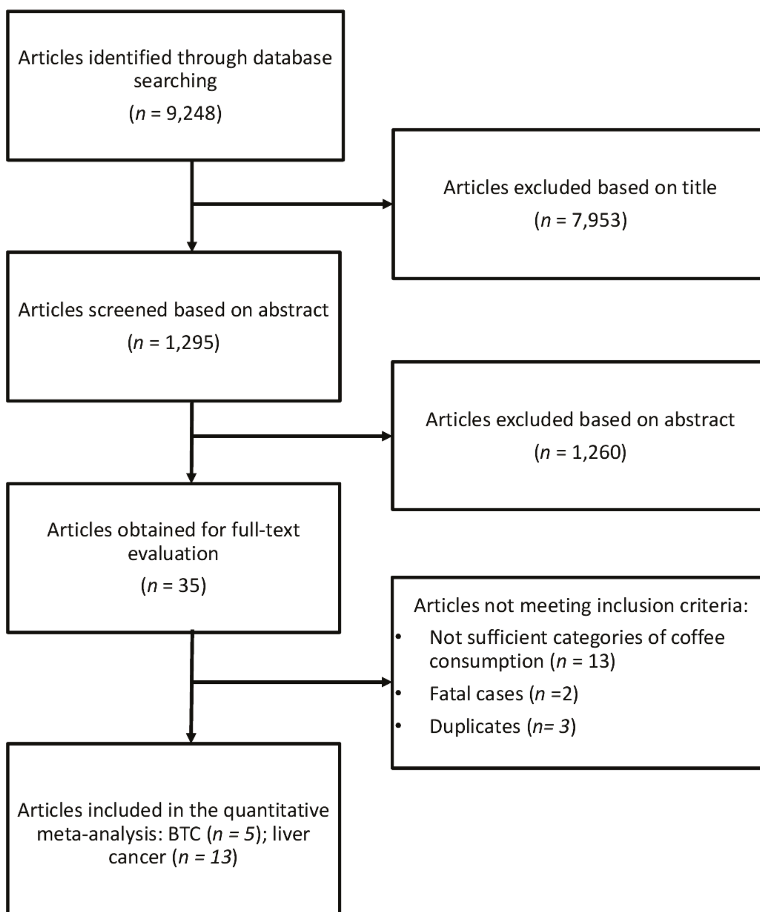


Figure 1. Flow chart and process selection of relevant studies exploring the association between coffee consumption and BTC and liver cancer risk.

Table 1. Characteristics of the studies included in the meta-analysis.

Author, Year	Cohort Name, Country	Years of Study, Follow-up	Cases; Controls/Total Population	Age Range, Gender	Adjustments
Biliary Tract Cancer					
Prospective					
Petrick, 2015	LCPP, USA	Multicentre	260; 1,212,893	MF	Age, sex, race, cohort, BMI, smoking status, cigarette smoking intensity, alcohol.
Makiuchi, 2016	JPHC, Japan	1990–2010 Cohort I, 1993–2010 Cohort II, 20 years (maximum) Cohort I, 17 years (maximum) Cohort II	267; 89,555	40–69 years, MF	Age, sex, study area, BMI, history of cholelithiasis, history of diabetes mellitus, history of chronic hepatitis or cirrhosis, history of smoking, drinking frequency, physical activity by METs/day score, total energy consumption, energy-adjusted consumption of fish, red meat, and vegetable and fruit, and green tea.
Larsson, 2017	SMC, COSM, Sweden	1998–2012, 13.3 years	74; 72,680	45–83 years, MF	Age, sex, education, smoking, BMI, and diabetes.
Case-Control					
Yen, 1987	USA	1975–1979, NA	67/272	50–79 years, MF	Sex and age in decades.
Chow, 1994	USA	1985–1989, NA	98/226	30–84 years, MF	Age, ethnic origin, and smoking status.
Liver Cancer					
Prospective					
Inoue, 2005	JPHC, Japan	1990–2001 Cohort I, 1993–2001 Cohort II, 9.7 years (average)	334; 90,452	40–69 years, MF	Sex, age, study area, tobacco-smoking status, ethanol intake, green vegetable intake, green tea drinking.
Hu, 2008	Finland	1972–2002, 19.3 years (average)	128; 60,323	25–74 years, MF	Age, sex, study year, alcohol consumption, education, smoking, diabetes and chronic liver disease at baseline and during follow-up, and BMI.
Inoue, 2009	JPHC, Japan	1993–2006 Cohort II, 12.7 years (average)	110; 18,815	40–69 years, MF	Sex, age, area, smoking status, weekly ethanol intake, BMI, history of diabetes mellitus, green tea consumption, serum ALT level, HCV infection status, and HBV infection status.
Johnson, 2011	SCHS, China	1993–2006, 13 years (maximum)	362; 61,321	45–74 years, MF	Age at recruitment, gender, dialect group, year of recruitment, BMI, level of education, consumption of alcoholic beverages, cigarette smoking, frequency of black tea and green tea intake, history of diabetes.
Lai, 2013	ATBC, Finland	1994–2009, 18.2 years (median)	194; 27,037	~57 years (median), M	ATBC intervention arm, age, BMI, education, marital status, history of diabetes, years of smoking, cigarettes smoked per day, alcohol, tea intake, and serum cholesterol.
Bamia, 2015	EPIC, Multicentre Europe	1992–2010, 11 years (median)	201; 486,799	25–70 years, MF	Age at recruitment, centre, sex, diabetes mellitus, education, BMI, smoking, physical activity, alcohol intake, energy intake, simultaneously including tea.

Table 1. *Cont.*

Author, Year	Cohort Name, Country	Years of Study, Follow-up	Cases; Controls/Total Population	Age Range, Gender	Adjustments
Petrick, 2015	LCPP, USA	Consortium (AARP, AHS, USRT, PLCO, WHS, CPSII, IWHS, BWHS, WHI)	860; 1,212,893	MF	Sex, age, race, cohort, BMI, smoking status, cigarette smoking intensity, alcohol.
Setiawan, 2015	MEC, USA	1993–2010, 18 years (median)	451; 162,022	45–75 years, MF	Age, sex, and race/ethnicity, education, BMI, alcohol intake, smoking status, and diabetes.
Case-Control					
Gallus, 2002	Greece, Italy	I study (Italy) 1984–1997, II study (Greece) 1995–1998, NA	333/360 Greece; 501/1552 Italy	20–79 years, MF	Age, sex, smoking, tobacco smoking, alcohol drinking, BMI, history of diabetes and hepatitis.
Gelatti, 2005	Italy	1994–2003, NA	250/500	50–79 years, MF	HBV infection, HCV infection, alcohol intake, sex and age.
Montella, 2007	Italy	1999–2002, NA	185/412	43–84 years, MF	Gender, age, centre, education, smoking habits, maximal lifetime alcohol intake and serological evidence of HCV and/or HBV infection.
Tanaka, 2007	Japan	2001–2004, NA	209/1308	40–79 years, MF	Sex, age, heavy alcohol use and smoking status.
Leung, 2011	China	2007–2008, NA	109/125	MF	Age, gender, cigarette smoking, alcohol use, tea consumption, physical activity.

Abbreviations: AARP: (American Association of Retired Persons) Diet and Health Study; AHS: Agricultural Health Study; ATBC: Alpha-Tocopherol, Beta-Carotene Cancer Prevention ATBC Study; BWHS: Black Women's Health Study; COSM: Cohort of Swedish Men; CPSII: Cancer Prevention Study II; EPIC: European Prospective Investigation into Cancer and Nutrition; IWHS: Iowa Women's Health Study; JPHC: Japan Public Health Center-based Prospective Study; LCPP: Liver Cancer Pooling Project; MEC: Multiethnic Cohort Study; PLCO: Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial; SCHS: Singapore Chinese Health Study; SMC: The Swedish Mammography Cohort; USRT: U.S. Radiologic Technologists (USRT) Cohort; WHI: Women's Health Initiative; WHS: Women's Health Study.

Thirteen studies [25,27–38], including seven studies on six prospective cohorts and one multicentre study (EPIC) [28–32,34,38], one pooling project of nine prospective cohorts [25], and five case-control studies [27,33,35–37], were eligible for the analysis on the association between coffee consumption and liver cancer. Eligible studies included 2,105,104 individuals and 4227 liver cancer cases. The main characteristics of the studies included in the meta-analysis are summarized in Table 1. Two studies were conducted in USA [25,34], six in Europe [27,28,31–33,36], and five in Asia [29,30,35,37,38]. The follow-up in prospective cohort studies ranged from about 9 to 19 years, and the age range at study baseline was 20–79 years.

3.2. Summary Relative Risk for the Highest versus Lowest Category of Coffee Consumption

The summary RR of BTC for the highest versus lowest category of coffee consumption was 0.83, 95% CI: 0.64, 1.08, with no evidence of heterogeneity I^2 = 0%, p = 0.58 (Figure 2). No publication bias was found after visual inspection of funnel plot (Figure S1). The pooled estimations were RR = 0.84, 95% CI: 0.61, 1.15; I^2 = 22%, p = 0.27 for prospective cohort studies, and RR = 0.74, 95% CI: 0.34, 1.63; I^2 = 0%, p = 0.82 for case-control studies (Figure 2). The subgroup analysis was not performed due to the limited number of studies eligible for the meta-analysis.

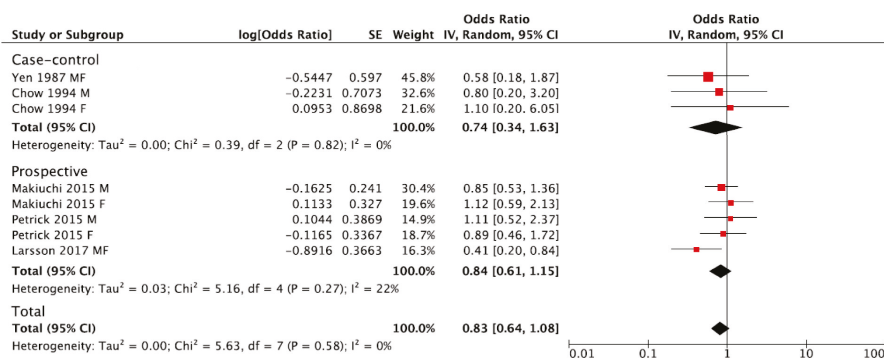


Figure 2. Forest plot of summary relative risks (RRs) of BTC for the highest versus lowest (reference) category of coffee consumption, by study design.

The summary RR of liver cancer for the highest versus lowest category of coffee consumption was RR = 0.52, 95% CI: 0.42, 0.63 with moderate heterogeneity I^2 = 44%, p = 0.02, (Figure 3). However, no publication bias was found after visual inspection of funnel plot (Figure S2). The summary RR in separate analysis for prospective cohort studies was RR = 0.53, 95% CI: 0.41, 0.69; I^2 = 46%, p = 0.03, and RR = 0.48, 95% CI: 0.33, 0.70; I^2 = 47%, p = 0.08 for case-control studies (Figure 3).

When considering sex and smoking status, no significant differences in comparison to main analysis of prospective cohorts were found (Table 2). In contrast, a significant decrease in risk of liver cancer for caffeinated coffee (RR = 0.65, 95% CI: 0.49, 0.86; I^2 = 0%, p = 0.59), but not for decaffeinated (RR = 0.85, 95% CI: 0.63, 1.14; I^2 = 0%, p = 0.96) was found (Table 2). In the stratified analysis, a lower risk of liver cancer was found among studies conducted in European and Asian countries compared to USA, even though all results were statistically significant (Table 2). Finally, stratified analysis by chronic hepatitis status did not significantly alter the results (Table 2).

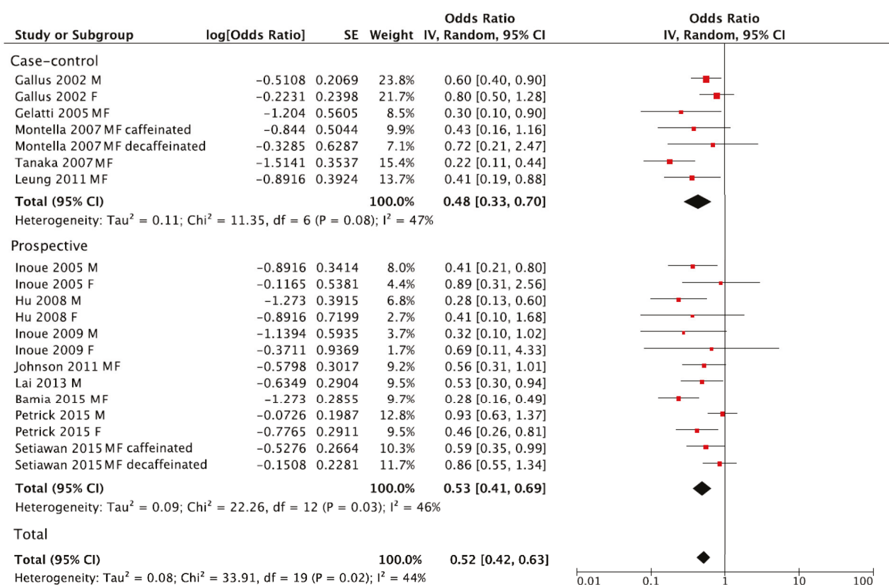


Figure 3. Forest plot of summary relative risks (RRs) of liver cancer for the highest versus lowest (reference) category of coffee consumption, by study design.

Table 2. Subgroup analyses of studies reporting risk of liver cancer for the highest versus lowest (reference) category of coffee consumption.

		Liver Cancer		
Subgroup	**No. of Datasets**	**RR (95% CI)**	I^2	$P_{heterogeneity}$
Total	20	0.52 (0.42, 0.63)	44%	0.02
Study design				
Prospective	13	0.53 (0.41, 0.69)	46%	0.03
Case-control	7	0.48 (0.33, 0.70)	47%	0.08
Gender				
Men				
Prospective	5	0.49 (0.30, 0.80)	64%	0.02
Case-control	1	0.60 (0.40, 0.80)	NA	NA
Women				
Prospective	4	0.53 (0.33, 0.83)	0%	0.71
Case-control	1	0.70 (0.50, 0.90)	NA	NA
Geographical location				
North America	4	0.72 (0.52, 0.98)	42%	0.16
Asia	7	0.42 (0.30, 0.58)	10%	0.35
Europe	9	0.48 (0.36, 0.64)	35%	0.14
Coffee type				
Caffeinated	3	0.65 (0.49, 0.86)	0%	0.59
Decaffeinated	4	0.85 (0.63, 1.14)	0%	0.96
Smoking status				
Never/former smoker	4	0.61 (0.43, 0.88)	32%	0.22
Current smoker	5	0.54 (0.36, 0.81)	61%	0.04
Chronic hepatitis				
Yes	7	0.56 (0.39, 0.80)	0%	0.87
No	5	0.60 (0.48, 0.75)	0%	0.71

3.3. Dose–Response Meta-Analysis

Three studies [23–25] were eligible for dose–response meta-analysis of prospective cohort studies on coffee consumption and BTC risk. In both non-linear and linear dose–response meta-analysis no significant association between coffee consumption and BTC risk was apparent (Figure 4, Table 3).

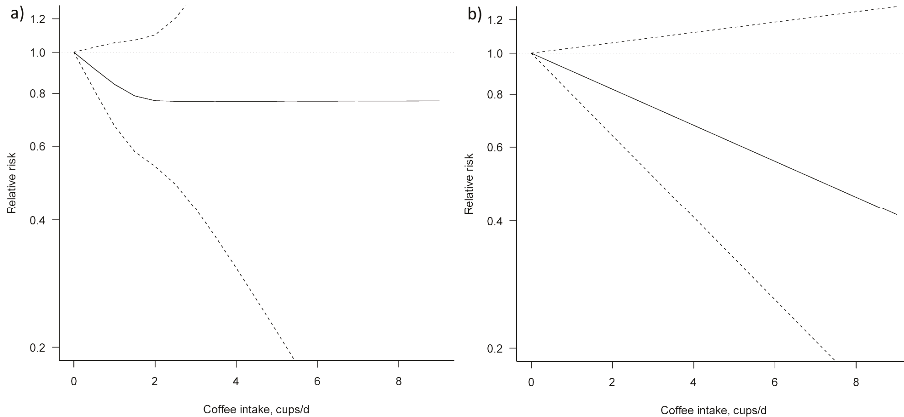


Figure 4. Dose–response association between coffee consumption and BTC risk (**a**) non-linear; (**b**) linear. Solid lines represent relative risk, dashed lines represent 95% confidence intervals.

For the dose–response analysis on the association between coffee consumption and liver cancer risk, seven studies were eligible [25,28–30,32,34,38]. We found an evidence of linear association between coffee consumption and liver cancer risk ($P_{for\ nonlinearity}$ = 0.954) (Figure 5, Table 4). Compared with no coffee consumption, the pooled relative risks for liver cancer were: 0.82, 95% CI: 0.70, 0.98 for one cup/day; 0.68, 95% CI: 0.53, 0.88 for two cups/day; 0.57, 95% CI: 0.46, 0.70 for three cups/day; 0.47, 95% CI: 0.39, 0.56 for four cups/day; 0.39, 95% CI: 0.31, 0.50 for five cups/day; 0.33, 95% CI: 0.23, 0.46 for six cups/day; and 0.27, 95% CI: 0.17, 0.43 for seven cups/day. The associations were similar for men and women, although, in the analysis for women, a higher heterogeneity ($P_{heterogeneity}$ = 0.692) was observed.

Table 3. Dose–response meta-analysis of prospective cohort studies on coffee consumption and biliary tract cancer risk.

	No. of Datasets (No. of Studies)	Coffee Intake (Cups/Day)								I^2 (%)	$P_{heterogeneity}$	$P_{non\text{-}linearity}$
		0	1	2	3	4	5	6	7			
Total analysis												
Non-linear	3 (3)	Reference	0.84 (0.67, 1.05)	0.77 (0.54, 1.10)	0.77 (0.43, 1.38)	0.77 (0.31, 1.91)	0.77 (0.22, 2.70)	0.77 (0.15, 3.86)	0.77 (0.11, 5.54)	0.54	0.15	0.46
Linear	3 (3)	Reference	0.91 (0.80, 1.03)	0.82 (0.64, 1.06)	0.75 (0.51, 1.09)	0.68 (0.41, 1.12)	0.61 (0.33, 1.15)	0.56 (0.26, 1.18)	0.50 (0.21, 1.22)	0.18	0.13	NA

Table 4. Dose–response meta-analysis of prospective cohort studies on coffee consumption and liver.

	No. of Datasets (No. of Studies)	Coffee Intake (Cups/Day)								I^2 (%)	$P_{heterogeneity}$	$P_{non\text{-}linearity}$
		0	1	2	3	4	5	6	7			
Total analysis												
Non-linear	7 (6)	Reference	0.82 (0.70, 0.98)	0.68 (0.53, 0.88)	0.57 (0.46, 0.7)	0.47 (0.39, 0.56)	0.39 (0.31, 0.5)	0.33 (0.23, 0.46)	0.27 (0.17, 0.43)	54.18	0.010	0.954
Linear	7 (6)	Reference	0.85 (0.82, 0.88)	0.72 (0.66, 0.78)	0.61 (0.54, 0.69)	0.52 (0.44, 0.61)	0.44 (0.36, 0.54)	0.58 (0.34, 0.98)	0.32 (0.24, 0.42)	17.54	0.296	NA
Male												
Non-linear	5 (5)	Reference	0.73 (0.57, 0.94)	0.56 (0.36, 0.85)	0.47 (0.30, 0.72)	0.42 (0.29, 0.60)	0.38 (0.27, 0.53)	0.33 (0.23, 0.46)	0.30 (0.19, 0.48)	72.9	0.000	0.286
Linear	4 (4)	Reference	0.84 (0.80, 0.89)	0.71 (0.64, 0.79)	0.60 (0.51, 0.71)	0.51 (0.41, 0.63)	0.43 (0.33, 0.56)	0.55 (0.47, 0.63)	0.31 (0.21, 0.44)	15.48	0.314	NA
Female												
Non-linear	4 (4)	Reference	0.87 (0.72, 1.06)	0.76 (0.56, 1.03)	0.65 (0.46, 0.92)	0.56 (0.31, 1.01)	0.48 (0.19, 1.22)	0.32 (0.22, 0.49)	0.35 (0.06, 1.90)	0	0.586	0.938
Linear	3 (3)	Reference	0.88 (0.80, 0.96)	0.77 (0.65, 0.92)	0.68 (0.52, 0.88)	0.59 (0.42, 0.84)	0.52 (0.34, 0.81)	0.53 (0.44, 0.65)	0.40 (0.22, 0.74)	0	0.692	NA

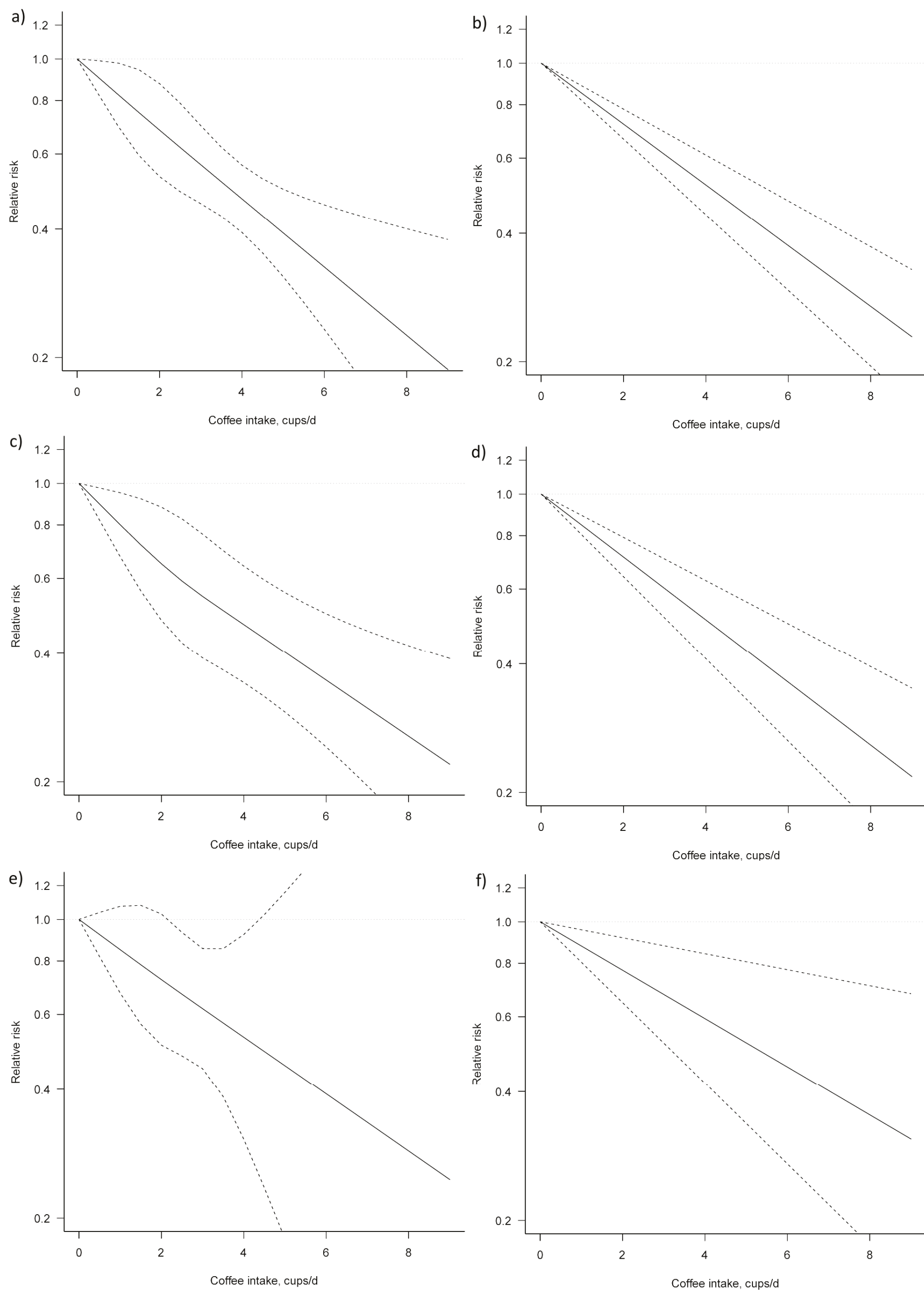


Figure 5. Dose–response association between coffee consumption and liver cancer risk: (**a**) non-linear, total analysis; (**b**) linear, total analysis; (**c**) non-linear, male; (**d**) linear, male; (**e**) non-linear, female; and (**f**) linear, female. Solid lines represent relative risk, while dashed lines represent 95% confidence intervals.

4. Discussion

In the present meta-analysis, the inverse association between coffee consumption and risk of liver cancer was consistent when taking into account key potential confounding factors. In contrast, no significant association between coffee consumption and risk of BTC was evident. Notably, a non-significant decreased risk was found especially for lower intake of coffee (i.e., two cups/day); however, higher intake was associated with no further benefit or rather an increased risk in two out of the three cohorts examined. Furthermore, the limited number of the studies eligible for meta-analysis is not sufficient to draw conclusions on the association between coffee consumption and BTC risk. From a mechanistic point of view, intake of both caffeinated and decaffeinated coffee stimulates gallbladder contraction caused by increased concentration in plasma cholecystokinin induced by coffee and decreases gallbladder volume by approximately 30% [9]. Furthermore, coffee can exert a protective effect on gallbladder by decreasing the crystallization of cholesterol in bile [10]. However, induction of gallbladder contraction in patients with gallstones may induce the passage of gallstones to bile duct [7]. Overall, whilst a rationale for potential benefit exists, findings to date do not support such hypotheses. A possible reason for heterogeneity between results could depend on the different population involved that may have different health risk behaviours. For instance, higher intake of coffee was relatively poorly associated with alcohol consumption in the Northern European cohorts [39], which showed a decreased risk of BTC. In contrast, coffee was associated with higher alcohol intake in Asian [40] and US cohorts [41–45], which reported no benefits of coffee consumption on BTC risk. However, current data are not sufficient to reach final conclusions and further investigations are needed to clarify the relation between coffee consumption and BTC taking into account potential confounders.

Findings on coffee consumption and liver cancer risk were more consistent: all sensitivity and subgroup analyses performed showed significant decreasing risk of cancer with a linear dose–response relation. Molecular targets involved in the chemopreventive effects of coffee include the nuclear factor E2-related factor 2 (Nrf2), responsible for transcription of enzymes involved in detoxification processes and in cellular antioxidant defences [46]: a diet rich in coffee has been demonstrated to increase gene expression of NAD(P)H: quinone oxidoreductase 1, glutathione *S*-transferase class Alpha 1, UDP-glucuronosyl transferase 1A6, and the glutamate cysteine ligase catalytic subunit, all involved in the antioxidant response of the organism [47]. With special regard to hepatocellular carcinoma, coffee decreased the incidence of liver tumours in rats [48], reduced the numbers of hyperplastic liver cell foci in chemical models of colon and liver cancer [49], and reduced solid tumour growth, proliferation, and hepatoma metastases [50,51].

A number of experimental studies provided the biological rationale for the components responsible for beneficial effects of coffee on liver cells. In this meta-analysis, a significant decrease in risk of liver cancer for caffeinated coffee, but not for decaffeinated, was found. Caffeine has been reported to reduce fibrosis in in vitro and animal studies, inhibiting TGF-beta-induced CTGF (Connective Tissue Growth Factor) expression in hepatocytes by stimulation of degradation of the TGF-beta effector SMAD 2, inhibition of SMAD3 phosphorylation and up-regulation of the PPARgamma-receptor [52,53], as well as increased activity of superoxide dismutase and catalase in the liver and increased expression of Nrf2 [54].

It has been shown that the caffeine metabolite paraxanthine may be responsible for the down-regulation of the expression of the fibrogenic protein CTGF in hepatic stellate cells and reduction of liver fibrosis and lipid peroxidation [55]. More recent investigations have shown that caffeine is not essential for the anti-fibrotic effects of coffee. It has been demonstrated in animal studies that both caffeinated and decaffeinated coffee reduce liver fibrosis and TGF-beta expression [56,57] and that use of decaffeinated coffee is able itself to reduce liver steatosis, inflammation and fibrosis in animal models [58]. The phenolic compounds chlorogenic acids and caffeic acid are among the main candidates for the antioxidant effects of coffee on liver. Chlorogenic acids administration, or treatment in animal studies, reduces liver fibrosis through decreased expression of collagen I and collagen III, as well as reducing the expression of inflammatory cytokines, TLR4, myeloid differentiation factor 88,

inducible nitric oxide synthase and cyclooxygenase-2 and nuclear factor-κB activation [59–61]. Caffeic acid reduces liver fibrosis due to its ability to suppress the activation of hepatic stellate cells by inhibiting oxidative stress through decrease of Keap1 expression, inhibition of Keap1 and Nrf2 binding, and thus activating Nrf2 and leading to increased expression of antioxidative signals [62–64]. Finally, coffee consumption may exert indirect protective effects on the liver due to the potential improvements of metabolism [3]. Coffee consumption has been inversely associated in several studies to metabolic syndrome [65–74], which has been related to liver fat accumulation and liver impairment due to common pathogenic determinants, such as insulin resistance and oxidative stress; impaired metabolism may induce progressive liver damage, liver inflammation and fibrosis, which ultimately may lead to carcinogenic transformation [75].

The results of the present study should be considered in the light of a number of limitations. First, some analyses reported moderate heterogeneity. As previously mentioned, several factors may explain differences across studies, including type of coffee bean (Arabica or Robusta), roasting, and beverage preparation. Secondly, genetic variants associated with caffeine metabolism are not considered in prospective cohort studies but were included in the meta-analysis and may contribute to the observed heterogeneity. Coffee consumption was assessed before outcome, thus recall bias is unlikely. However, misclassification of the actual amounts consumed may have affected the dose–response relation. Reverse causation may have affected the results if individuals changed coffee intake due to a diagnosed medical condition or disease; however, any such effects would be muted in studies with a long duration.

5. Conclusions

In conclusion, coffee may represent a valid functional food for liver protection. Current evidence is sufficient to guide future clinical randomized trials to test the hepatoprotective effects of coffee, which in turn may lead to more definitive recommendations. However, further observational studies with better in depth analyses of potential confounding factors are needed to test the association between coffee consumption and BTC.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/9/950/s1, Table S1: Meta-Analysis of Observational Studies in Epidemiology (MOOSE) checklist, Table S2: Search strategy, Table S3: Excluded studies, Figure S1: Funnel plot for BTC risk of the highest versus lowest (reference) category of coffee consumption, Figure S2: Funnel plot liver cancer risk of the highest versus lowest (reference) category of coffee consumption).

Author Contributions: J.G. designed the study, performed search and analysis; A.M. performed search and analysis; F.S. and M.M. provided insights on the topic; and J.G., D.D.R. and S.R. drafted the paper. All authors critically revised the papers.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, caffeine, and health outcomes: An umbrella review. *Annu. Rev. Nutr.* **2017**, *37*, 131–156. [CrossRef] [PubMed]
2. Grosso, G.; Micek, A.; Godos, J.; Sciacca, S.; Pajak, A.; Martinez-Gonzalez, M.A.; Giovannucci, E.L.; Galvano, F. Coffee consumption and risk of all-cause, cardiovascular, and cancer mortality in smokers and non-smokers: A dose-response meta-analysis. *Eur. J. Epidemiol.* **2016**, *31*, 1191–1205. [CrossRef] [PubMed]
3. Buscemi, S.; Marventano, S.; Antoci, M.; Cagnetti, A.; Castorina, G.; Galvano, F.; Marranzano, M.; Mistretta, A. Coffee and metabolic impairment: An updated review of epidemiological studies. *NFS J.* **2016**, *3*, 1–7. [CrossRef]
4. Caprioli, G.; Cortese, M.; Sagratini, G.; Vittori, S. The influence of different types of preparation (espresso and brew) on coffee aroma and main bioactive constituents. *Int. J. Food Sci. Nutr.* **2015**, *66*, 505–513. [CrossRef] [PubMed]
5. Koksal, E.; Yardimci, H.; Kocaadam, B.; Deniz Gunes, B.; Yilmaz, B.; Karabudak, E. Relationship between dietary caffeine intake and blood pressure in adults. *Int. J. Food Sci. Nutr.* **2016**, *68*, 1–7. [CrossRef] [PubMed]

6. Godos, J.; Pluchinotta, F.R.; Marventano, S.; Buscemi, S.; Li Volti, G.; Galvano, F.; Grosso, G. Coffee components and cardiovascular risk: Beneficial and detrimental effects. *Int. J. Food Sci. Nutr.* **2014**, *65*, 925–936. [CrossRef] [PubMed]
7. Espinoza, J.A.; Bizama, C.; Garcia, P.; Ferreccio, C.; Javle, M.; Miquel, J.F.; Koshiol, J.; Roa, J.C. The inflammatory inception of gallbladder cancer. *Biochim. Biophys. Acta* **2016**, *1865*, 245–254. [CrossRef] [PubMed]
8. Hsing, A.W.; Gao, Y.T.; Han, T.Q.; Rashid, A.; Sakoda, L.C.; Wang, B.S.; Shen, M.C.; Zhang, B.H.; Niwa, S.; Chen, J.; et al. Gallstones and the risk of biliary tract cancer: A population-based study in China. *Br. J. Cancer* **2007**, *97*, 1577–1582. [CrossRef] [PubMed]
9. Douglas, B.R.; Jansen, J.B.; Tham, R.T.; Lamers, C.B. Coffee stimulation of cholecystokinin release and gallbladder contraction in humans. *Am. J. Clin. Nutr.* **1990**, *52*, 553–556. [PubMed]
10. Lillemoe, K.D.; Magnuson, T.H.; High, R.C.; Peoples, G.E.; Pitt, H.A. Caffeine prevents cholesterol gallstone formation. *Surgery* **1989**, *106*, 400–407. [PubMed]
11. Marventano, S.; Salomone, F.; Godos, J.; Pluchinotta, F.; Del Rio, D.; Mistretta, A.; Grosso, G. Coffee and tea consumption in relation with non-alcoholic fatty liver and metabolic syndrome: A systematic review and meta-analysis of observational studies. *Clin. Nutr.* **2016**, *35*, 1269–1281. [CrossRef] [PubMed]
12. Liu, F.; Wang, X.; Wu, G.; Chen, L.; Hu, P.; Ren, H.; Hu, H. Coffee consumption decreases risks for hepatic fibrosis and cirrhosis: A meta-analysis. *PLoS ONE* **2015**, *10*, e0142457. [CrossRef]
13. Kennedy, O.J.; Roderick, P.; Buchanan, R.; Fallowfield, J.A.; Hayes, P.C.; Parkes, J. Systematic review with meta-analysis: Coffee consumption and the risk of cirrhosis. *Aliment. Pharmacol. Ther.* **2016**, *43*, 562–574. [CrossRef]
14. Bravi, F.; Tavani, A.; Bosetti, C.; Boffetta, P.; La Vecchia, C. Coffee and the risk of hepatocellular carcinoma and chronic liver disease: A systematic review and meta-analysis of prospective studies. *Eur. J. Cancer Prev.* **2016**, *26*, 368–377. [CrossRef] [PubMed]
15. Bai, K.; Cai, Q.; Jiang, Y.; Lv, L. Coffee consumption and risk of hepatocellular carcinoma: A meta-analysis of eleven epidemiological studies. *Onco Targets Ther.* **2016**, *9*, 4369–4375. [PubMed]
16. Stroup, D.F.; Berlin, J.A.; Morton, S.C.; Olkin, I.; Williamson, G.D.; Rennie, D.; Moher, D.; Becker, B.J.; Sipe, T.A.; Thacker, S.B. Meta-analysis of observational studies in epidemiology: A proposal for reporting, Meta-analysis of observational studies in epidemiology (moose) group. *JAMA* **2000**, *283*, 2008–2012. [CrossRef] [PubMed]
17. Wells, G.A.; Shea, B.; O'Connell, D.; Peterson, J.; Welch, V.; Losos, M.; Tugwell, P. *The Newcastle-Ottawa Scale (NOS) for Assessing the Quality of Nonrandomised Studies in Meta-Analyses*; Ottawa Health Research Institute: Ottawa, ON, Canada, 1999.
18. Greenland, S. Quantitative methods in the review of epidemiologic literature. *Epidemiol. Rev.* **1987**, *9*, 1–30. [CrossRef] [PubMed]
19. Greenland, S.; Longnecker, M.P. Methods for trend estimation from summarized dose-response data, with applications to meta-analysis. *Am. J. Epidemiol.* **1992**, *135*, 1301–1309. [CrossRef] [PubMed]
20. Orsini, N.; Bellocco, R.; Greenland, S. Generalized least squares for trend estimation of summarized dose-response data. *Stata J.* **2006**, *6*, 40–57.
21. Orsini, N.; Li, R.; Wolk, A.; Khudyakov, P.; Spiegelman, D. Meta-analysis for linear and nonlinear dose-response relations: Examples, an evaluation of approximations, and software. *Am. J. Epidemiol.* **2012**, *175*, 66–73. [CrossRef]
22. Chow, W.H.; McLaughlin, J.K.; Menck, H.R.; Mack, T.M. Risk factors for extrahepatic bile duct cancers: Los Angeles county, California (USA). *Cancer Causes Control* **1994**, *5*, 267–272. [CrossRef] [PubMed]
23. Larsson, S.C.; Giovannucci, E.L.; Wolk, A. Coffee consumption and risk of gallbladder cancer in a prospective study. *J. Natl. Cancer Inst.* **2017**, *109*, 1–3. [CrossRef]
24. Makiuchi, T.; Sobue, T.; Kitamura, T.; Ishihara, J.; Sawada, N.; Iwasaki, M.; Sasazuki, S.; Yamaji, T.; Shimazu, T.; Tsugane, S. Association between green tea/coffee consumption and biliary tract cancer: A population-based cohort study in Japan. *Cancer Sci.* **2016**, *107*, 76–83. [CrossRef] [PubMed]
25. Petrick, J.L.; Freedman, N.D.; Graubard, B.I.; Sahasrabuddhe, V.V.; Lai, G.Y.; Alavanja, M.C.; Beane-Freeman, L.E.; Boggs, D.A.; Buring, J.E.; Chan, A.T.; et al. Coffee consumption and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma by sex: The liver cancer pooling project. *Cancer Epidemiol. Biomark. Prev.* **2015**, *24*, 1398–1406. [CrossRef] [PubMed]

26. Yen, S.; Hsieh, C.C.; MacMahon, B. Extrahepatic bile duct cancer and smoking, beverage consumption, past medical history, and oral-contraceptive use. *Cancer* **1987**, *59*, 2112–2116. [CrossRef]
27. Gallus, S.; Bertuzzi, M.; Tavani, A.; Bosetti, C.; Negri, E.; La Vecchia, C.; Lagiou, P.; Trichopoulos, D. Does coffee protect against hepatocellular carcinoma? *Br. J. Cancer* **2002**, *87*, 956–959. [CrossRef] [PubMed]
28. Hu, G.; Tuomilehto, J.; Pukkala, E.; Hakulinen, T.; Antikainen, R.; Vartiainen, E.; Jousilahti, P. Joint effects of coffee consumption and serum gamma-glutamyltransferase on the risk of liver cancer. *Hepatology* **2008**, *48*, 129–136. [CrossRef] [PubMed]
29. Inoue, M.; Kurahashi, N.; Iwasaki, M.; Shimazu, T.; Tanaka, Y.; Mizokami, M.; Tsugane, S. Japan Public Health Center-Based Prospective Study, G. Effect of coffee and green tea consumption on the risk of liver cancer: Cohort analysis by hepatitis virus infection status. *Cancer Epidemiol. Biomark. Prev.* **2009**, *18*, 1746–1753. [CrossRef]
30. Inoue, M.; Yoshimi, I.; Sobue, T.; Tsugane, S.; Group, J.S. Influence of coffee drinking on subsequent risk of hepatocellular carcinoma: A prospective study in Japan. *J. Natl. Cancer Inst.* **2005**, *97*, 293–300. [CrossRef] [PubMed]
31. Lai, G.Y.; Weinstein, S.J.; Albanes, D.; Taylor, P.R.; McGlynn, K.A.; Virtamo, J.; Sinha, R.; Freedman, N.D. The association of coffee intake with liver cancer incidence and chronic liver disease mortality in male smokers. *Br. J. Cancer* **2013**, *109*, 1344–1351. [CrossRef] [PubMed]
32. Bamia, C.; Lagiou, P.; Jenab, M.; Trichopoulou, A.; Fedirko, V.; Aleksandrova, K.; Pischon, T.; Overvad, K.; Olsen, A.; Tjonneland, A.; et al. Coffee, tea and decaffeinated coffee in relation to hepatocellular carcinoma in a European population: Multicentre, prospective cohort study. *Int. J. Cancer* **2015**, *136*, 1899–1908. [CrossRef] [PubMed]
33. Montella, M.; Polesel, J.; La Vecchia, C.; Dal Maso, L.; Crispo, A.; Crovatto, M.; Casarin, P.; Izzo, F.; Tommasi, L.G.; Talamini, R.; et al. Coffee and tea consumption and risk of hepatocellular carcinoma in Italy. *Int. J. Cancer* **2007**, *120*, 1555–1559. [CrossRef] [PubMed]
34. Setiawan, V.W.; Wilkens, L.R.; Lu, S.C.; Hernandez, B.Y.; Le Marchand, L.; Henderson, B.E. Association of coffee intake with reduced incidence of liver cancer and death from chronic liver disease in the US multiethnic cohort. *Gastroenterology* **2015**, *148*, 118–125. [CrossRef] [PubMed]
35. Tanaka, K.; Hara, M.; Sakamoto, T.; Higaki, Y.; Mizuta, T.; Eguchi, Y.; Yasutake, T.; Ozaki, I.; Yamamoto, K.; Onohara, S.; et al. Inverse association between coffee drinking and the risk of hepatocellular carcinoma: A case-control study in Japan. *Cancer Sci.* **2007**, *98*, 214–218. [CrossRef] [PubMed]
36. Gelatti, U.; Covolo, L.; Franceschini, M.; Pirali, F.; Tagger, A.; Ribero, M.L.; Trevisi, P.; Martelli, C.; Nardi, G.; Donato, F.; et al. Coffee consumption reduces the risk of hepatocellular carcinoma independently of its aetiology: A case-control study. *J. Hepatol.* **2005**, *42*, 528–534. [CrossRef] [PubMed]
37. Leung, W.W.; Ho, S.C.; Chan, H.L.; Wong, V.; Yeo, W.; Mok, T.S. Moderate coffee consumption reduces the risk of hepatocellular carcinoma in hepatitis b chronic carriers: A case-control study. *J. Epidemiol. Community Health* **2011**, *65*, 556–558. [CrossRef] [PubMed]
38. Johnson, S.; Koh, W.P.; Wang, R.; Govindarajan, S.; Yu, M.C.; Yuan, J.M. Coffee consumption and reduced risk of hepatocellular carcinoma: Findings from the singapore chinese health study. *Cancer Causes Control* **2011**, *22*, 503–510. [CrossRef] [PubMed]
39. Larsson, S.C.; Giovannucci, E.; Wolk, A. Coffee consumption and stomach cancer risk in a cohort of Swedish women. *Int. J. Cancer* **2006**, *119*, 2186–2189. [CrossRef] [PubMed]
40. Kurahashi, N.; Inoue, M.; Iwasaki, M.; Sasazuki, S.; Tsugane, S. Japan Public Health Center Study, G. Coffee, green tea, and caffeine consumption and subsequent risk of bladder cancer in relation to smoking status: A prospective study in Japan. *Cancer Sci.* **2009**, *100*, 294–291. [CrossRef]
41. Boggs, D.A.; Palmer, J.R.; Stampfer, M.J.; Spiegelman, D.; Adams-Campbell, L.L.; Rosenberg, L. Tea and coffee intake in relation to risk of breast cancer in the black women's health study. *Cancer Causes Control* **2010**, *21*, 1941–1948. [CrossRef] [PubMed]
42. Dominianni, C.; Huang, W.Y.; Berndt, S.; Hayes, R.B.; Ahn, J. Prospective study of the relationship between coffee and tea with colorectal cancer risk: The plco cancer screening trial. *Br. J. Cancer* **2013**, *109*, 1352–1359. [CrossRef] [PubMed]
43. Gierach, G.L.; Freedman, N.D.; Andaya, A.; Hollenbeck, A.R.; Park, Y.; Schatzkin, A.; Brinton, L.A. Coffee intake and breast cancer risk in the nih-aarp diet and health study cohort. *Int. J. Cancer* **2012**, *131*, 452–460. [CrossRef] [PubMed]

44. Groessl, E.J.; Allison, M.A.; Larson, J.C.; Ho, S.B.; Snetslaar, L.G.; Lane, D.S.; Tharp, K.M.; Stefanick, M.L. Coffee consumption and the incidence of colorectal cancer in women. *J. Cancer Epidemiol.* **2016**, *2016*, 6918431. [CrossRef] [PubMed]
45. Pereira, M.A.; Parker, E.D.; Folsom, A.R. Coffee consumption and risk of type 2 diabetes mellitus: An 11-year prospective study of 28 812 postmenopausal women. *Arch. Intern. Med.* **2006**, *166*, 1311–1316. [CrossRef] [PubMed]
46. Cavin, C.; Marin-Kuan, M.; Langouet, S.; Bezencon, C.; Guignard, G.; Verguet, C.; Piguet, D.; Holzhauser, D.; Cornaz, R.; Schilter, B. Induction of NRF2-mediated cellular defenses and alteration of Phase I activities as mechanisms of chemoprotective effects of coffee in the liver. *Food Chem. Toxicol.* **2008**, *46*, 1239–1248. [CrossRef] [PubMed]
47. Higgins, L.G.; Cavin, C.; Itoh, K.; Yamamoto, M.; Hayes, J.D. Induction of cancer chemopreventive enzymes by coffee is mediated by transcription factor NRF2. Evidence that the coffee-specific diterpenes cafestol and kahweol confer protection against acrolein. *Toxicol. Appl. Pharmacol.* **2008**, *226*, 328–337. [CrossRef] [PubMed]
48. Tanaka, T.; Nishikawa, A.; Shima, H.; Sugie, S.; Shinoda, T.; Yoshimi, N.; Iwata, H.; Mori, H. Inhibitory effects of chlorogenic acid, reserpine, polyprenoic acid (e-5166), or coffee on hepatocarcinogenesis in rats and hamsters. *Basic Life Sci.* **1990**, *52*, 429–440. [PubMed]
49. Mori, H.; Tanaka, T.; Shima, H.; Kuniyasu, T.; Takahashi, M. Inhibitory effect of chlorogenic acid on methylazoxymethanol acetate-induced carcinogenesis in large intestine and liver of hamsters. *Cancer Lett.* **1986**, *30*, 49–54. [CrossRef]
50. Miura, Y.; Furuse, T.; Yagasaki, K. Inhibitory effect of serum from rats administered with coffee on the proliferation and invasion of rat ascites hepatoma cells. *Cytotechnology* **1997**, *25*, 221–225. [CrossRef] [PubMed]
51. Miura, Y.; Ono, K.; Okauchi, R.; Yagasaki, K. Inhibitory effect of coffee on hepatoma proliferation and invasion in culture and on tumor growth, metastasis and abnormal lipoprotein profiles in hepatoma-bearing rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* **2004**, *50*, 38–44. [CrossRef] [PubMed]
52. Chan, E.S.; Montesinos, M.C.; Fernandez, P.; Desai, A.; Delano, D.L.; Yee, H.; Reiss, A.B.; Pillinger, M.H.; Chen, J.F.; Schwarzschild, M.A.; et al. Adenosine a(2a) receptors play a role in the pathogenesis of hepatic cirrhosis. *Br. J. Pharmacol.* **2006**, *148*, 1144–1155. [CrossRef] [PubMed]
53. Gressner, O.A.; Lahme, B.; Rehbein, K.; Siluschek, M.; Weiskirchen, R.; Gressner, A.M. Pharmacological application of caffeine inhibits tgf-beta-stimulated connective tissue growth factor expression in hepatocytes via ppargamma and smad2/3-dependent pathways. *J. Hepatol.* **2008**, *49*, 758–767. [CrossRef] [PubMed]
54. Gordillo-Bastidas, D.; Oceguera-Contreras, E.; Salazar-Montes, A.; Gonzalez-Cuevas, J.; Hernandez-Ortega, L.D.; Armendariz-Borunda, J. NRF2 and snail-1 in the prevention of experimental liver fibrosis by caffeine. *World J. Gastroenterol.* **2013**, *19*, 9020–9033. [CrossRef] [PubMed]
55. Gressner, O.A.; Lahme, B.; Siluschek, M.; Gressner, A.M. Identification of paraxanthine as the most potent caffeine-derived inhibitor of connective tissue growth factor expression in liver parenchymal cells. *Liver Int.* **2009**, *29*, 886–897. [CrossRef] [PubMed]
56. Arauz, J.; Moreno, M.G.; Cortes-Reynosa, P.; Salazar, E.P.; Muriel, P. Coffee attenuates fibrosis by decreasing the expression of TGF-beta and CTGF in a murine model of liver damage. *J. Appl. Toxicol.* **2013**, *33*, 970–979. [CrossRef] [PubMed]
57. Furtado, K.S.; Prado, M.G.; Aguiar, E.S.M.A.; Dias, M.C.; Rivelli, D.P.; Rodrigues, M.A.; Barbisan, L.F. Coffee and caffeine protect against liver injury induced by thioacetamide in male wistar rats. *Basic Clin. Pharmacol. Toxicol.* **2012**, *111*, 339–347. [CrossRef] [PubMed]
58. Vitaglione, P.; Morisco, F.; Mazzone, G.; Amoruso, D.C.; Ribecco, M.T.; Romano, A.; Fogliano, V.; Caporaso, N.; D'Argenio, G. Coffee reduces liver damage in a rat model of steatohepatitis: The underlying mechanisms and the role of polyphenols and melanoidins. *Hepatology* **2010**, *52*, 1652–1661. [CrossRef] [PubMed]
59. Shi, H.; Dong, L.; Bai, Y.; Zhao, J.; Zhang, Y.; Zhang, L. Chlorogenic acid against carbon tetrachloride-induced liver fibrosis in rats. *Eur. J. Pharmacol.* **2009**, *623*, 119–124. [CrossRef] [PubMed]
60. Shi, H.; Dong, L.; Jiang, J.; Zhao, J.; Zhao, G.; Dang, X.; Lu, X.; Jia, M. Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway. *Toxicology* **2013**, *303*, 107–114. [CrossRef]

61. Monente, C.; Ludwig, I.A.; Stalmach, A.; de Pena, M.P.; Cid, C.; Crozier, A. In vitro studies on the stability in the proximal gastrointestinal tract and bioaccessibility in caco-2 cells of chlorogenic acids from spent coffee grounds. *Int. J. Food Sci. Nutr.* **2015**, *66*, 657–664. [CrossRef] [PubMed]
62. Li, M.; Wang, X.F.; Shi, J.J.; Li, Y.P.; Yang, N.; Zhai, S.; Dang, S.S. Caffeic acid phenethyl ester inhibits liver fibrosis in rats. *World J. Gastroenterol.* **2015**, *21*, 3893–3903. [CrossRef] [PubMed]
63. Pang, C.; Zheng, Z.; Shi, L.; Sheng, Y.; Wei, H.; Wang, Z.; Ji, L. Caffeic acid prevents acetaminophen-induced liver injury by activating the keap1-NRF2 antioxidative defense system. *Free Radic. Biol. Med.* **2016**, *91*, 236–246. [CrossRef] [PubMed]
64. Yazgan, U.C.; Elbey, B.; Kus, S.; Baykal, B.; Keskin, I.; Yilmaz, A.; Sahin, A. Effect of caffeic acid phenethyl ester on oxidant and anti-oxidant status of liver and serum in a rat model with acute methanol intoxication. *Ir. J. Med. Sci.* **2016**, *186*, 519–523. [CrossRef]
65. Dos Santos, P.R.; Ferrari, G.S.; Ferrari, C.K. Diet, sleep and metabolic syndrome among a legal amazon population, Brazil. *Clin. Nutr. Res.* **2015**, *4*, 41–45. [CrossRef] [PubMed]
66. Driessen, M.T.; Koppes, L.L.; Veldhuis, L.; Samoocha, D.; Twisk, J.W. Coffee consumption is not related to the metabolic syndrome at the age of 36 years: The amsterdam growth and health longitudinal study. *Eur. J. Clin. Nutr.* **2009**, *63*, 536–542. [CrossRef] [PubMed]
67. Chang, C.S.; Chang, Y.F.; Liu, P.Y.; Chen, C.Y.; Tsai, Y.S.; Wu, C.H. Smoking, habitual tea drinking and metabolic syndrome in elderly men living in rural community: The tianliao old people (top) study 02. *PLoS ONE* **2012**, *7*, e38874. [CrossRef] [PubMed]
68. Grosso, G.; Marventano, S.; Galvano, F.; Pajak, A.; Mistretta, A. Factors associated with metabolic syndrome in a mediterranean population: Role of caffeinated beverages. *J. Epidemiol.* **2014**, *24*, 327–333. [CrossRef]
69. Grosso, G.; Stepaniak, U.; Micek, A.; Topor-Madry, R.; Pikhart, H.; Szafraniec, K.; Pajak, A. Association of daily coffee and tea consumption and metabolic syndrome: Results from the polish arm of the hapiee study. *Eur. J. Nutr.* **2015**, *54*, 1129–1137. [CrossRef] [PubMed]
70. Lutsey, P.L.; Steffen, L.M.; Stevens, J. Dietary intake and the development of the metabolic syndrome: The atherosclerosis risk in communities study. *Circulation* **2008**, *117*, 754–761. [CrossRef] [PubMed]
71. Matsuura, H.; Mure, K.; Nishio, N.; Kitano, N.; Nagai, N.; Takeshita, T. Relationship between coffee consumption and prevalence of metabolic syndrome among Japanese civil servants. *J. Epidemiol.* **2012**, *22*, 160–166. [CrossRef] [PubMed]
72. Nordestgaard, A.T.; Thomsen, M.; Nordestgaard, B.G. Coffee intake and risk of obesity, metabolic syndrome and type 2 diabetes: A mendelian randomization study. *Int. J. Epidemiol.* **2015**, *44*, 551–565. [CrossRef] [PubMed]
73. Takami, H.; Nakamoto, M.; Uemura, H.; Katsuura, S.; Yamaguchi, M.; Hiyoshi, M.; Sawachika, F.; Juta, T.; Arisawa, K. Inverse correlation between coffee consumption and prevalence of metabolic syndrome: Baseline survey of the Japan Multi-Institutional Collaborative Cohort (J-MICC) study in Tokushima, Japan. *J. Epidemiol.* **2013**, *23*, 12–20. [CrossRef] [PubMed]
74. Suliga, E.; Koziel, D.; Ciesla, E.; Rebak, D.; Gluszek, S. Coffee consumption and the occurrence and intensity of metabolic syndrome: A cross-sectional study. *Int. J. Food Sci. Nutr.* **2016**, *68*, 1–7. [CrossRef] [PubMed]
75. Salomone, F.; Galvano, F.; Li Volti, G. Molecular bases underlying the hepatoprotective effects of coffee. *Nutrients* **2017**, *9*, 85. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Effects of Simulated Human Gastrointestinal Digestion of Two Purple-Fleshed Potato Cultivars on Anthocyanin Composition and Cytotoxicity in Colonic Cancer and Non-Tumorigenic Cells

Stan Kubow [1,*], Michèle M. Iskandar [1], Emiliano Melgar-Bermudez [1], Lekha Sleno [2], Kebba Sabally [1], Behnam Azadi [1], Emily How [1], Satya Prakash [3], Gabriela Burgos [4] and Thomas zum Felde [4,*]

[1] School of Human Nutrition, McGill University, 21,111 Lakeshore, Ste. Anne de Bellevue, QC H9X 3V9, Canada; michele.iskandar@mail.mcgill.ca (M.M.I.); emiliano.melgar-bermudez@mail.mcgill.ca (E.M.-B.); kebba.sabally@mcgill.ca (K.S.); behnam.azadi@mcgill.ca (B.A.); emily.how@mail.mcgill.ca (E.H.)

[2] Chemistry Department, Université du Québec à Montréal, P.O. Box 8888, Downtown Station, Montreal, QC H3C 3P8, Canada; sleno.lekha@uqam.ca

[3] BioMedical Engineering Department, McGill University, 3775 University Street, Room 311, Montreal, QC H3A 2B4, Canada; satya.prakash@mcgill.ca

[4] International Potato Center (CIP), Avenida La Molina 1895, Apartado Postal 1558, Lima, Lima 12, Peru; g.burgos@cgiar.org

* Correspondence: stan.kubow@mcgill.ca (S.K.); t.zumfelde@cgiar.org (T.z.F.); Tel.: +1-514-398-7754 (S.K.); +51-1-349-6017-3053 (T.z.F.); Fax: +1-514-398-7739 (S.K.); +51-1-317-5326 (T.z.F.)

Received: 22 June 2017; Accepted: 24 August 2017; Published: 29 August 2017

Abstract: A dynamic human gastrointestinal (GI) model was used to digest cooked tubers from purple-fleshed Amachi and Leona potato cultivars to study anthocyanin biotransformation in the stomach, small intestine and colonic vessels. Colonic Caco-2 cancer cells and non-tumorigenic colonic CCD-112CoN cells were tested for cytotoxicity and cell viability after 24 h exposure to colonic fecal water (FW) digests (0%, 10%, 25%, 75% and 100% FW in culture media). After 24 h digestion, liquid chromatography-mass spectrometry identified 36 and 15 anthocyanin species throughout the GI vessels for Amachi and Leona, respectively. The total anthocyanin concentration was over thirty-fold higher in Amachi compared to Leona digests but seven-fold higher anthocyanin concentrations were noted for Leona versus Amachi in descending colon digests. Leona FW showed greater potency to induce cytotoxicity and decrease viability of Caco-2 cells than observed with FW from Amachi. Amachi FW at 100% caused cytotoxicity in non-tumorigenic cells while FW from Leona showed no effect. The present findings indicate major variations in the pattern of anthocyanin breakdown and release during digestion of purple-fleshed cultivars. The differing microbial anthocyanin metabolite profiles in colonic vessels between cultivars could play a significant role in the impact of FW toxicity on tumor and non-tumorigenic cells.

Keywords: purple-fleshed potato; anthocyanins; biotransformation; human gastrointestinal model; antioxidant; cancer cells; cytotoxicity

1. Introduction

Potatoes are a versatile sustainable food staple in many countries providing populations with an affordable source of key phytonutrients and valuable income for growers. Colored fleshed potatoes contain anthocyanins, which are red, blue and purple water soluble pigments that occur in the glycosylated form with one or more sugar moieties [1]. Anthocyanins are glycosides or acylglycosides

of six common dietary aglycone anthocyanidins, which consist of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Anthocyanin structures vary by glycosidic substitution and acylation of the sugar groups with acids that can include acetic acid, *p*-coumaric acid, caffeic acid, malonic acid, sinapic acid, ferulic acid, oxalic acid and succinic acid [2]. Glucose, xylose, galactose, arabinose and rhamnose are common monosaccharides in anthocyanins attached to the C3 position of the flavan structure, whereas common diglycosides found in anthocyanins include rutinose (glucose and rhamnose) and sophorose (glucose and glucose).

There is great interest in the food content of anthocyanins due to their proposed chemopreventative properties towards several disease processes such as atherosclerosis and retinal light-induced damage [1]. Anthocyanins are present in the flesh and skin of several purple and red fleshed potatoes, which show a wide range of anthocyanin structures and concentrations that are largely cultivar-dependent [3] and location-dependent [4]. The anthocyanin content of colored fleshed potatoes has been related to a two- to three-fold greater antioxidant potential than white-fleshed potatoes [1,3]. Boiled purple-fleshed potatoes are a good source of anthocyanins and show high antioxidant capacity [1], although the effect of boiling on anthocyanin content can also be variety-dependent [5]. Since potato is an important food staple that is considered the most important vegetable crop worldwide, the contribution of potatoes towards anthocyanin intake can be significant.

There is fundamental information lacking regarding how the anthocyanin structures and bioactivities are affected during digestion of pigmented potatoes. Such knowledge can ultimately lead to enhancement of the chemopreventative properties of colored fleshed potatoes for consumers via breeding practices favoring genetic variants with desirable anthocyanin profiles. Anthocyanins are released from the food matrix and undergo biotransformation during the gastrointestinal digestive processes involving pH changes and digestive enzymes. In addition, they undergo major degradation via their metabolism by colonic microflora [6]. Simulated gastrointestinal digestion studies that include microbial batch cultures demonstrate major decreases in anthocyanins after exposure to pancreatic enzymes with an additional decline in anthocyanins after human fecal microbial metabolism [7,8]. Computer-controlled dynamic multistage continuous digestion models involving the ascending colon, transverse colon and descending colon vessels more closely simulate in vivo conditions due to pH adjustment of each colonic bioreactor, allowing for the development of varying microbial communities with different metabolic activities in each colonic vessel [9–11]. Varying results have been observed with respect to antioxidant capacity measures following simulated gut digestion of anthocyanin-rich foods with reports showing either a diminishment [12] or enhancement [13] of antioxidant activity post-digestion. Simulated digestion studies of anthocyanin-rich purple-fleshed potatoes that include the microbial and digestive conditions associated with different colonic segments of the gastrointestinal tract have not been performed to examine changes in anthocyanin structure or antioxidant capacity.

Dietary anthocyanins are implicated in the protective effects of fruits and vegetables against cancer. Anthocyanin-rich fractions and extracts from blueberry [14], bilberries [15,16], blackberries [17], chokecherry [18], java plum [19], sweet potato [20] and tart cherries [21] have been shown to effectively inhibit the growth of a variety of human colonic cancer cell lines. Extracts of anthocyanin-containing tart cherries [21,22], black raspberries [23] and purple corn [24] have been demonstrated to also inhibit tumor development in different animal colon cancer models. In terms of anthocyanin-rich potatoes, extracts from Bora Valley, Purple Majesty, Mountain Rose, Northstar and the wild species *Solanum pinnatisectum* exhibited inhibition of the growth of cultured human malignant cells [25]. The anti-proliferative properties of purple-fleshed potato extracts were also seen in HCT-116 and HT-29 colon cancer lines, regardless of prior baking or chip processing [26]. Despite such encouraging findings there has been limited research regarding how anti-carcinogenic activity is affected by changes in anthocyanin structures caused by digestive processes. A recent study showed that pepsin-pancreatin digests of the high anthocyanin-containing cv. Vitelotte noire purple potatoes were associated with diminished cell viability in the Caco-2 colon cancer cell model [27]. To our knowledge, the anti-cancer

impact of colonic microbial anthocyanin metabolites generated from the digestion of anthocyanin-rich foods has not been previously addressed.

The first objective of this study was to evaluate the biotransformation of anthocyanins in cooked samples of the two purple-fleshed potato cultivars Amachi and Leona after digestion in the Computer Controlled Dynamic Human Gastrointestinal Model (GI model). Samples of both cultivars underwent digestion via the GI model and liquid chromatography-electrospray ionization-time-of-flight (LC-ESI-TOF) mass spectrometry (MS) was used to assess anthocyanin profiles and antioxidant capacity measures following digestive processes in compartments of the GI model (stomach, small intestine, ascending, transverse and descending colon). The second objective was to compare the effects of FW digests of the two cultivars obtained from the colonic reactors from the GI model on the cytotoxicity and cell viability on the human colonic adenocarcinoma Caco-2 cell line and normal colonic epithelial cells (CCD-112CoN).

2. Materials and Methods

2.1. Plant Material

Tubers from two intensive purple-fleshed cultivars, Amachi and Leona (Figure 1), grown in Andahuaylas, Apurimac, Peru, were used in this study. Andahuaylas is located at 2926 meters above sea level in the Peruvian Andes. Both cultivars were selected based on their high total anthocyanin content (360 mg/100 g and 180 mg/100 g, respectively) and high antioxidant activity (945 mg Trolox equivalent/100 g and 542 mg Trolox equivalent/100 g, respectively) expressed on a fresh weight basis [28]. One hundred tubers from each cultivar were processed by the Quality and Nutrition Laboratory at the International Potato Center (CIP) in Lima, Peru, where representative tubers were cooked, peeled, freeze dried and milled through 40 mesh following the procedure described by Porras et al., 2014 [29]. Freeze dried and milled samples of each cultivar were sent to the School of Human Nutrition, McGill University, Canada.

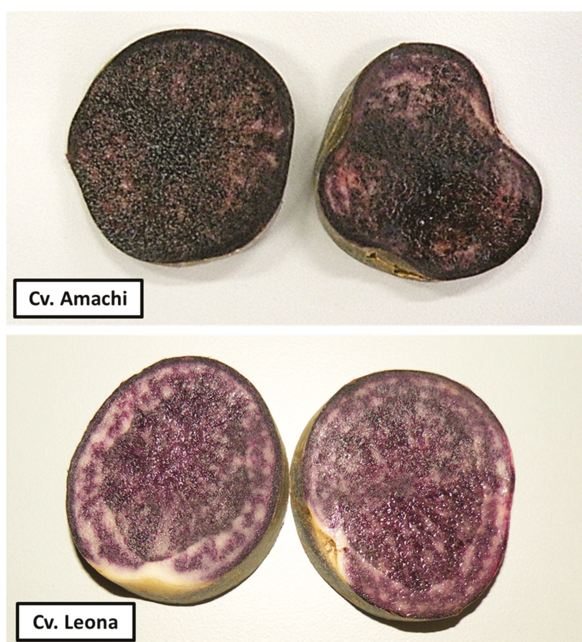


Figure 1. The purple-fleshed potato cultivars Amachi and Leona.

2.2. Computer Controlled Dynamic Human Gastrointestinal Model

The simulated human GI model consisted of five consecutive reactors that represent the stomach (V1), small intestine (V2), the ascending (V3), the transverse (V4) and the descending colon (V5) that are interconnected by plastic tubing and peristaltic pumps as previously described [11]. The system is fully computer-controlled (LabVIEW® software, National Instruments, Austin, TX, USA) for the addition of food to V1 and buffers to adjust pH of all compartments and pancreatic juice to V2. The pH was measured with a probe connected to a pH meter and was automatically adjusted to keep a pH of 2.0 in V1 and 6.5 in V2 via addition of 0.2 M NaOH or 0.5 M HCl. The flow of intestinal content between reactors was automatically computer controlled with a transit time of 2 h in each of the V1 and V2 compartments followed by 4 h digestion in the colonic vessels. The volume in the V1 and V2 reactors was 200 mL and the V3, V4 and V5 reactors had volumes of 500, 800 and 600 mL, respectively. Temperature-controlled water flowed between the double glass jacketed reactors to keep the temperature at 37 °C. The passage of food in the stomach was simulated by the addition of gastric solution including 0.1 M HCl and pepsin (P7125, Sigma-Aldrich, Oakville, ON, Canada). Upon entering V2, pancreatic juice supplemented with bile (12 g/L NaHCO, 0.9 g/L pancreatin; P 1750, Sigma-Aldrich) and 6 g/L Oxgall (DF 0128-17-8, Fisher Scientific, Ottawa, ON, Canada) were added to neutralize stomach acidity. The colonic vessels V3, V4 and V5 were pH-controlled between 5.6 and 5.9; 6.1 and 6.4; and 6.6 and 6.9, respectively. Freshly collected fecal samples from five non-smoking, healthy volunteers (3 males, 2 females aged 30–65 with BMI between 20 and 24.99 kg/m^2) with no history of GI disease or antibiotic use in the previous 6 months were pooled and used to prepare a 10% fresh fecal slurry in sterile phosphate-buffered saline solution that inoculated the three colonic reactors. The fermentation vessels were kept at 37 °C and were stirred continuously on magnetic stirrers. The colonic reactors were maintained anaerobic by daily purging for 20 min into the headspace with oxygen-free nitrogen gas. For the bacterial populations from the fecal slurry to stabilize, the system was allowed to run for two weeks before the addition of the potato meal (stabilization period). During this time and during potato meal digestion, the system was supplied with 300 mL of GI nutrient solution at 8-h intervals, or three times daily. Prior to use, the solution was adjusted to pH 2, autoclaved, and stored at 37 °C. The composition of the GI nutrient solution was composed of arabinogalactan (1 g/L), pectin (2 g/L), xylan (1 g/L), glucose (0.4 g/L), yeast extracts (3 g/L), peptone (1 g/L), mucin (4 g/L), all of which were purchased from Sigma-Aldrich; starch (3 g/L) and cysteine powders (0.5 g/L), both of which were purchased from Fisher Scientific; this nutrient solution was previously shown to stabilize the microbial community in the colonic vessels [11]. This approach has been validated using enumeration procedures, short chain fatty acid production patterns, enzymatic activities, gas production, and by microorganism-associated activities [9–11].

After the 2-week stabilization period, the potato meal digestion was started in the GI model that consisted of 18.5 g of the cooked, freeze-dried, and milled phenolic-rich cultivars Amachi and Leona. On the day of treatment, the freeze-dried potato tuber samples were incorporated into the GI food solution and subjected to three 8 h cycles of digestion by the GI model. The calculated amount of potato meal provided to the gut model was based on the dry matter content of cooked freeze-dried potato (25.36%) according to the United States Department of Agriculture food database. The meal provided approximately one-half of the single serving of potato, which reflects typical daily intake for one cultivar as part of a daily mixed potato cultivar intake in Peru (80 kg/year) [30].

Aliquots (20 mL) were collected from all the vessels of the GI model before addition of the freeze-dried potato meal (t = 0 control) and after 8 h (t = 8) and 24 h (t = 24) of digestion. One day of treatment was followed by a 3-day washout period when the system was fed the control GI nutrient solution without potato. FW was prepared from the aliquots via centrifugation at 200× g for 20 min and the supernatants were stored at −80 °C for later analysis. To prevent photodecomposition, all digestive compartments and collection vessels were wrapped in tin foil.

2.3. LC-ESI-TOF-MS Analysis

Samples were thawed, spiked with 10 μg/mL keracyanin chloride internal standard, vortexed, filtered with 25 mm syringe filters (0.2 μm, MCE, sterile; 09-719C, Fisher Scientific) into high-performance liquid chromatography (HPLC) vials for ESI-TOF-MS analysis, which was conducted based on a modified method of Tian et al., 2005 [31]. Sample analysis was carried out using an Agilent 1200 series HPLC system equipped with an Agilent 6210 (LC-ESI-TOF)-(MS) (Agilent, Santa Clara, CA, USA). Anthocyanins were separated using gradient conditions on a Gemini-NX column (5 μm, 100 mm × 4.6 mm) (Phenomenex, Torrance, CA, USA). Elution involved mobile phases A (water + 0.1% formic acid (FA)) and B (acetonitrile + 0.1% FA). Initially, the mobile phase composition was held at 5% B for 1 min, then increased to 25% B at 30 min, with a sharp increase to 80% at 31 min held for 3 additional minutes prior to re-equilibration time back at the initial conditions. Solvent flow rate of 1 mL/min was used and 20 μL of sample was injected. Accurate mass data were obtained by both positive and negative ion ESI, which involved injection via two different methods. Data was acquired over a mass (m) to charge (z) ratio (m/z) range of 100 to 1000. The settings of tuning parameters were: gas flow 12 L/min, temperature 350 °C, capillary voltage (+/−) 4000 V, skimmer voltage 60 V, fragmentor 100 V and nebulizer 50 psi (344.74 kPa). The reference masses for internal calibration of the high resolution mass spectra were m/z: 121.050873, 922.009798 for the positive mode and m/z: 119.03632, 966.000725 for the negative mode. The acquisition of mass spectra was carried out using the MassHunter acquisition software (Agilent Technologies, software version 4.01b) and further processing was done using MassHunter Qualitative Analysis. Anthocyanins in the samples were identified based on their masses and information obtained from the literature. Anthocyanins were quantified using an internal standard keracyanin chloride (cyanidin-3-O-rutinoside chloride) and anthocyanin concentrations were expressed as keracyanin chloride equivalents (KCC-Eq). Extracted ion chromatograms of accurate masses for protonated (MH^+) ions were used for confirmation of presence of parent anthocyanin compounds as well as metabolites within ± 10 ppm.

2.4. Ferric Reducing Antioxidant Power Assay

The ferric reducing antioxidant power (FRAP) assay described by Benzie and Strain (1996) [32] was used to determine the total antioxidant potential of the supernatant of collected FW samples. The FRAP reagent was prepared in a 10:1:1 ratio of 300 mM acetate buffer (pH 3.6) of 10 mM 2,4,6-tripyridyl-s-triazine solution, 40 mM HCl at 50 °C, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. Once the FRAP working solution was prepared it was immediately incubated for 10 min at 37 °C. The reaction was carried out using a 96-well plate filled with 10 μL of sample or standard, 30 μL of H_2O and 200 μL of the FRAP working solution for 30 min. The absorbance was read at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA). Ferrous sulfate solution was used as an external standard with a calibration curve range of 0.1 to 10 mM. The results were expressed as ferrous sulfate equivalents.

2.5. Cytotoxic Effects of FW with and without Purple Potato Digests on Caco-2 and CCD112-CoN Cells

The colonic adenocarcinoma Caco-2 cells and CCD-112CoN cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cell culture medium was obtained from Invitrogen (Carlsbad, CA, USA). Caco-2 cells were cultured in Dulbecco's modified Eagle's Minimum Essential Medium (EMEM, pH 7.4) supplemented with 20% heat inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin. CCD-112CoN cells were cultured in EMEM and 10% FBS. Both cell lines were grown in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. For cytotoxicity experiments, Caco-2 cells and CCD-112CoN cells were seeded on 24-well plates at cell densities of 5.0×10^5 and 7.6×10^4 cells per well, respectively [33] and incubated under the same atmospheric conditions for 24 h or until confluent. The cells were then treated with either FW alone or FW containing in vitro digests of the cvs. Amachi and Leona. The digests were pooled from the

ascending, transverse and descending colon vessels from the human simulated gut model in the studies described above. Digests were filter-sterilized and the pH was adjusted to 7 using 0.1 N HCl or 0.1 N NaOH. The digests were mixed with EMEM (2% FBS) at six treatment concentrations (0%, 10%, 25%, 75% and 100% FW in a total volume of 500 μL per well) that were administered to the cells for 24 h. The 0% dose consisted of EMEM (2% FBS) only. Lactate dehydrogenase (LDH) released into the culture medium was used as an indicator of cell viability. Cell-free supernatants were collected and LDH was assessed using the Cytotoxicity Detection Kit (Roche Diagnostics, Laval, QC, Canada) according to the manufacturer's instructions. LDH production from damaged cells was expressed as percent of LDH produced by untreated cells. LDH release is a reliable method for assessment of the extent of cell death regardless of type of cell death [34]. To compare the effects of the two cultivars on tumor cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [35] was performed with Caco-2 cells. Briefly, the cells were washed with PBS following the 24 h incubation with the digests and incubated for 3 h with MTT solution (0.5 mg/mL). The purple formazan crystals produced by viable metabolically active cells were then dissolved using a lysis solution (0.4 M HCl in 100% isopropanol) and absorbance was measured at 540 nm. Cell viability was expressed as percent of untreated control. Cell viability values regarding FW from the two cultivars were also compared based on IC_{50} values. Each treatment was administered in duplicate and four independent experiments were conducted. Statistical analyses were performed using SigmaPlot v. 13 (Systat Software Inc., Chicago, IL, USA). The cytotoxic effects of cvs. Amachi and Leona in vitro digests on the two cell lines were analyzed by two-way analysis of variance (ANOVA) using dose and cell line as main factors followed by Tukey's post-hoc test for multiple comparisons. For the MTT assay, data were analyzed by two-way ANOVA using cultivar and dose as main factors followed by Tukey's post-hoc test for multiple comparisons. The IC_{50} values were compared using Student's *t*-test. Data are represented as mean of four independent experiments ± SE. Differences were considered to be significant at $p < 0.05$.

3. Results

3.1. Anthocyanins in Digested Amachi and Leona Cultivars

The majority of anthocyanins in potatoes have been shown to possess the structure: anthocyanidin 3-acyl-rutinoside-5-glucoside. The most common acyl substitutes are caffeoyl, *p*-coumaroyl or feruloyl residues [36,37]. This is in agreement with the data in Table 1 where these anthocyanin structures were predominant in V1 for both tested potato cultivars.

The total anthocyanin content measured throughout the GI model vessels differed greatly between the two cultivars as Amachi contained greater than 30-fold higher content. The overall percent composition of anthocyanin concentrations of Amachi in the GI model consisted of 70% petunidins, 20.8% peonidins, 8.9% cyanidins and 0.3% pelargonidins. In contrast to the predominant presence of petunidin in Amachi, the most abundant anthocyanins in Leona were cyanidin based compounds constituting 48.9% of the species found throughout the gastrointestinal model, followed by a 17.8% of peonidin based compounds, 17.7% pelargonidins and 15.6% petunidins.

Table 1. Proposed identification of anthocyanin peaks in pigmented potato cvs. Amachi and Leona and their concentration in the samples exposed to human simulated intestinal digestion (mg/L) [1].

Measured Accurate Mass			Amachi					Leona				
(*m/z*) [2]	Proposed Compound [3]	RT	V1	V2	V3	V4	V5	V1	V2	V3	V4	V5
287.06	Cyanidin [4]	3.1	0.38	-	-	-	-	0.40	-	-	0.31	0.48
301.03	Peonidin [5]	4.8	0.47	-	0.35	-	-	-	-	-	-	-
433.11	Pelargonidin 3-glucoside	18	-	-	-	0.06	0.17	-	-	0.05	0.13	0.25
463.12	Petunidin 3-rhamnoside	18	-	-	-	-	-	-	-	0.03	0.10	0.16
583.14	Peonidin 3-*p*-hydroxybenzoyl-glucoside	5	0.16	-	-	-	-	0.07	0.14	0.14	0.22	-
610.16	Pelargonidin 3-feruloyl-glucoside	15	0.12	-	-	-	-	0.31	0.20	0.13	0.10	0.04
611.14	Cyanidin 3-(6-caffeoyl-glucoside)	15	-	-	0.17	-	-	-	0.09	0.02	0.03	0.04
611.16	Cyanidin 3-sophoroside	15	-	0.10	0.15	-	-	-	0.12	0.02	0.04	0.07
612.14	Peonidin 3-(6-*p*-coumaroyl-glucoside)	3.6	-	-	0.09	-	-	0.09	0.07	-	-	-
625.15	Peonidin 3-(6-caffeoyl-glucoside)	13	2.3	-	-	-	-	-	-	-	-	-
625.17	Petunidin 3-rutinoside	13	2.6	-	-	-	-	-	-	-	-	-
731.16	Cyanidin 3-(6-caffeoyl-6-*p*-hydroxybenzoyl-glucoside)	10	3.53	-	0.62	-	-	0.96	-	-	-	-
731.18	Cyanidin 3-*p*-hydroxybenzoyl-sophoroside	10	3.20	-	1.14	-	-	0.96	-	-	-	-
757.19	Cyanidin 3-*p*-coumaroyl-sophoroside	7	0.26	-	-	-	-	-	-	-	-	-
757.21	Pelargonidin 3-sophoroside-5-glucoside	7	0.28	-	-	-	-	0.07	-	-	-	-
771.21	Peonidin 3-(6″-*p*-coumaryl-sophoroside)	9	1.14	-	-	-	-	0.16	-	0.08	-	-
773.19	Cyanidin 3-(6″-caffeoyl-sophoroside)	15	-	-	0.53	-	-	-	-	-	-	-
773.21	Cyanidin 3-sophoroside-5-glucoside	7	-	-	-	1.11	-	-	-	-	-	-
787.20	Peonidin 3-(6″-caffeoyl-sophoroside)	7.7	4.55	-	-	0.23	-	0.26	-	-	-	-
787.22	Petunidin 3-rutinoside-5-glucoside	7.7	6.12	-	-	0.34	-	0.26	-	-	-	-
801.20	Peonidin 3-caffeoyl-feruloyl-glucoside	10	0.22	-	-	-	-	-	-	-	-	-
801.22	Peonidin 3-(6″-feruloyl-sophoroside)	10	0.30	0.07	-	-	-	-	-	-	-	-
893.21	Cyanidin 3-(6″-caffeoyl-6″-*p*-hydroxybenzoyl-sophoroside)	11	-	-	0.39	0.08	-	-	-	-	-	-
893.23	Cyanidin 3-*p*-hydroxybenzoyl-sophoroside-5-glucoside	11	-	-	0.39	0.07	-	-	-	-	-	-
903.23	Cyanidin 3-(6′,6″-dicoumaroyl-sophoroside)	20	2.20	-	-	-	-	-	-	-	-	-
907.22	Peonidin 3-caffeoyl-*p*-hydroxybenzoyl-sophoroside	13	-	-	0.04	0.02	-	-	-	-	-	-
907.25	Peonidin 3-*p*-hydroxybenzoyl-sophoroside-5-glucoside	20	-	-	0.02	-	-	-	-	-	-	-
917.27	Peonidin 3-*p*-coumaroyl-rutinoside-5-glucoside	22	16.42	-	-	-	-	0.06	-	-	-	-
919.25	Cyanidin 3-*p*-coumaroyl-sophoroside-5-glucoside	18	2.53	-	-	-	-	-	-	-	-	-
920.23	Cyanidin 3-caffeoyl-*p*-coumaroyl-sophoroside	18	1.11	-	-	-	-	-	-	-	-	-
931.25	Cyanidin 3-feruloyl-sophoroside-5-glucoside	20	0.09	-	-	-	-	-	-	-	-	-
933.26	Petunidin 3-*p*-coumaroyl-rutinoside-5-glucoside	20	132.79	0.40	0.07	0.01	0.02	0.15	0.13	-	0.04	0.26
949.23	Peonidin 3-dicaffeoyl-sophoroside	18	3.64	-	-	-	-	-	-	-	-	-
949.26	Peonidin 3-(6‴-caffeoyl-sophoroside)-5-glucoside	18	3.47	-	-	-	-	-	-	-	-	-
963.25	Peonidin 3-caffeoyl-feruloyl-sophoroside	20	4.39	-	-	-	-	-	-	-	-	-
963.27	Peonidin 3-(6″-feruloyl-sophoroside)-5-glucoside	20	4.45	-	-	-	-	-	-	-	-	-
	Total Anthocyanins Measured		196.72	0.57	3.96	1.92	0.19	3.75	0.75	0.47	0.97	1.3

[1] Expressed as cyanidin 3-rutinoside equivalents after spiking samples with 10 μg/mL of keracyanin chloride. [2] Determined by liquid chromatography-electrospray ionization-time-of-flight analysis. [3] Identification based on previous literature data. [4] Cyanidin other RT 27 (Leona V4 and Leona V5). [5] Peonidin other RT 3.7 in Amachi V3.

The anthocyanin compound clearly identified to be present at the highest concentration in V1 for Amachi was petanin (petunidin 3-*p*-coumaroyl-rutinoside-5-glucoside (*m*/*z* 933)), which was found at concentrations several-fold higher than any other anthocyanin accounting for 67.5% of the total anthocyanin content. The relatively high abundance of petanin in the Amachi cv. has been previously reported in other pigmented potato cultivars including Hermanns Blaue, Highland Burgundy Red, Shetland Black and Vitelotte [38] as well as four purple or dark fleshed tetraploid Andigenum potato cultivars [36]. The presence of the aglycones cyanidin (*m*/*z* 287.01), peonidin (*m*/*z* 301.03) and petunidin (*m*/*z* 301.07) [36] in V1 of Amachi corresponds to their presence noted previously in purple potatoes [36,39]. Cyanidin was also noted in V1 for the Leona sample and accounted for 21% of the total anthocyanin content in the GI model. Peonanin (peonidin-3-*p*-coumaroyl-rutinoside-5-glucoside (*m*/*z* 917.27) and petunidin-3-rutinoside-5-glucoside (*m*/*z* 787.22) that were noted in V1 of the Amachi and Leona cultivars have been identified in purple-fleshed cultivars [36,39,40].

For Amachi, the concentration of most anthocyanin species was found to be highest in V1, which contained 97% of the total anthocyanins found throughout the GI model. The aglycones cyanidin and petunidin were only found in V1, while peonidin was observed in both V1 and V3. The Leona cv. contrasted greatly with the Amachi cv. in terms of the distribution of anthocyanin species in the GI model as only 46% of the total anthocyanins were present in the V1 vessel. The remaining anthocyanins were present primarily in the V4 and V5 colonic vessels that comprised 35% of the total anthocyanins. For both cultivars, a biphasic pattern in anthocyanin concentrations was observed in terms of lowered anthocyanin concentrations in V2 followed by increased amounts in the subsequent colonic vessels, which has been previously seen during anthocyanin digestion [41,42]. Anthocyanins are likely to be unstable in the V2 intestinal vessel due to their chemical decomposition at neutral pH [43], which is known to occur before subsequent exposure to colonic microbial metabolism [44]. Degradation of anthocyanins that has ranged from 30 to 80% has been reported in pancreatic in vitro digestion studies [7,45]. Significant anthocyanin losses after intestinal digestion have been observed previously with simulated GI digestion model studies of plant foods such as mulberry molasses and pestil [46] and red cabbage [8].

While most anthocyanins glycosylated at 3′O and 5′O in Amachi were present in V1, pelargonidin 3-glucoside (*m*/*z* 433.11), cyanidin 3-sophoroside-5-glucoside (*m*/*z* 773.21) and cyanidin 3-*p*-hydroxybenzoyl-sophoroside-5-glucoside (*m*/*z* 893.23) were only present in other vessels, which could indicate that their release from the food matrix occurs later in digestion. There were six other species that were not seen in the V1 vessel for Amachi, which included cyanidin 3-(6-caffeoyl-glucoside) (*m*/*z* 611.14), cyanidin 3-sophoroside (*m*/*z* 611.16), peonidin 3-(6-*p*-coumaroyl-glucoside) (*m*/*z* 612.14), cyanidin 3-(6″-caffeoyl-sophoroside) (*m*/*z* 773.19), cyanidin 3-(6″-caffeoyl-6″-*p*-hydroxybenzoyl-sophoroside) (*m*/*z* 893.21) and peonidin 3-caffeoyl-*p*-hydroxybenzoyl-sophoroside (*m*/*z* 907.22). These species predominantly first appeared in the V3 ascending colon vessel, which suggests microbial metabolism provided for the release of these anthocyanins from the Amachi potato food matrix. Similarly, four anthocyanins were not noted in V1 for the Leona sample, which were pelargonidin 3-glucoside (*m*/*z* 433.11), petunidin 3-rhamnoside (*m*/*z* 463.12), cyanidin 3-(6-caffeoyl-glucoside) (*m*/*z* 611.14) and cyanidin 3-sophoroside (*m*/*z* 611.16). Interestingly, despite the overall several-fold higher total anthocyanin concentrations in the GI model vessels for the Amachi versus Leona cultivar, a seven-fold higher anthocyanin content in the Leona V5 was observed as compared to V5 anthocyanin concentrations for the Amachi cv. The above findings signify major differences in anthocyanin breakdown and release among the various digestive compartments between the two purple-fleshed potato cultivars. Such findings might be due to several cultivar matrix-mediated effects including: (a) enzymatic release of anthocyanins from the food matrix; (b) enzymatic and microbial-facilitated degradation of anthocyanins; (c) de-conjugation and subsequent re-conjugation of anthocyanins; (d) release and re-incorporation of anthocyanins within the fecal matrix; and (e) presence of other dietary constituents released during digestion on anthocyanin solubility.

Differentiating between different isobaric compounds could be done by looking at the retention time of the compound in some cases; for example, peonidin-3-caffeoyl-*p*-hydroxybenzoyl-sophoroside (m/z 907.22) was found to have a retention time of 13, while peonidin 3-*p*-hydroxybenzoyl-sophoroside 5-glucoside (m/z 907.25) was found at a retention time of 20. In other cases, isobaric compounds are not present always in the same vessels, potentially indicating that the method used for separation was accurate enough to isolate compounds that had very similar MW, such as cyanidin 3-sophoroside (MW: 611.16) and cyanidin 3-(6″-caffeoyl-glucoside) (MW: 611.14); the former is present in both the Amachi samples vessels V2 and V3, while the latter is only present in V3.

3.2. Antioxidant Activity

Samples were withdrawn from each of the five vessels of the GI model at t = 0 (before addition of potato meal) and t = 8 h and 24 h of digestion and antioxidant activity was assessed using the FRAP assay. As shown in Figure 2, antioxidant activity increased in vessels V1–V3 at 8 h, and further increased at 24 h of digestion. During exposure to intestinal V2 digestion, however, a decrease from initial values in the stomach V1 vessel was observed with both cultivars. This latter result coincides with previous studies showing a decrease in FRAP capacity of fresh apple [47] and anthocyanin-rich extracts and raw red cabbage shown after pepsin and pancreatin-bile digestion [8]. The antioxidant power of Amachi in all gut model vessels was approximately double that of Leona, which could be partly related to the several-fold higher content of both anthocyanins and chlorogenic acid (see Supplementary Materials, Figure S1) of Amachi in comparison to Leona. Degradation of the parent polyphenolic compounds including anthocyanins was likely responsible for the observed drop in antioxidant capacity seen in V2 vs. V1. A further decrease in antioxidant capacity was observed in V3, which was likely due to further diminishment of parent compounds resulting from gut microbial metabolism to generate secondary phenolic metabolites.

An increase in FRAP antioxidant activity in the V4 and V5 colonic reactors was only apparent at t = 24 h for the Amachi and this latter increase was only seen in V4 for Leona. Metabolic microbial breakdown of anthocyanins over a 24 h period appears to generate sufficient amounts of microbial metabolites to produce an improvement in antioxidant capacity. The latter result is supportive of the concept that microbial metabolites generated from ingested polyphenols can contribute to antioxidant capacity of human fecal fluid. In support of this contention, previous in vitro digestion studies have noted that a decrease in anthocyanin content is accompanied by an increase in antioxidant activity, which has been related to an increase in the concentrations of smaller molecular weight antioxidant phenolics [48]. The appearance of anthocyanin species in distal vessels of the GI model of both cultivars (Table 1) also supports this concept. Anthocyanins and their metabolites can, via antioxidant activity, provide protection of intestinal cells against oxidative stress in the gut, and hence alleviate gut inflammation [49], protect against colorectal cancer and generally enhance colorectal health [50]. It has been shown that the radical scavenging antioxidant ability of feces from healthy human subjects is 20-fold higher than that of plasma [51]. Desirable physiological effects following absorption may also be exerted by secondary metabolites generated from microbial polyphenol degradation. For instance, in a study where healthy volunteers consumed pomegranate juice for five days, microbial metabolites of polyphenols were detected in the plasma and urine, but the parent polyphenols were not [52].

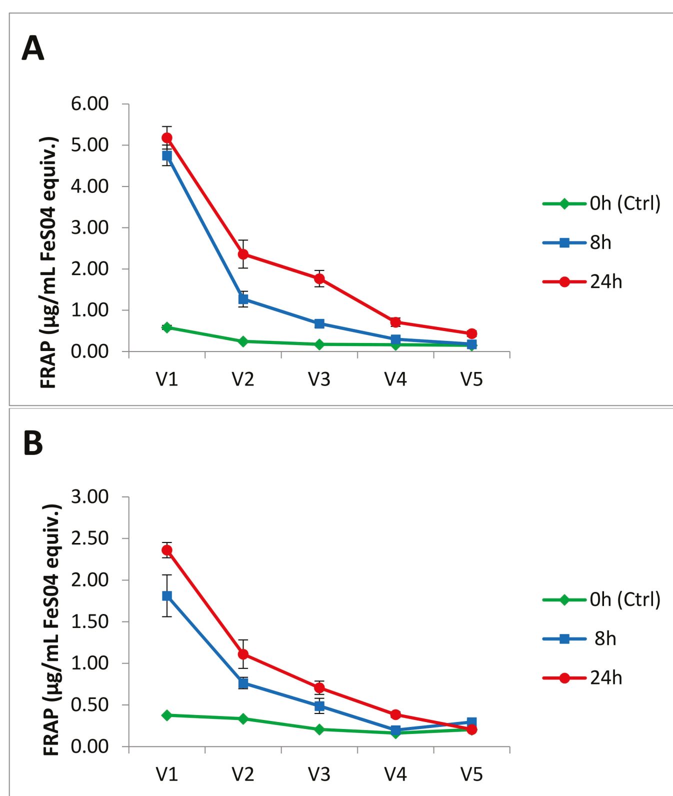


Figure 2. Time course of antioxidant capacity ferric reducing antioxidant power measures of digesta from gut model vessels following provision of a meal containing cvs. Amachi (**A**) and Leona (**B**) potato meals.

3.3. Cytotoxicity and Cell Viability in Caco-2 and CCD-112CoN Cells

Two human intestinal cell lines (the colorectal adenocarcinoma Caco-2 cell line and the normal colonic CCD-112CoN cell line) were used to evaluate the cytotoxic effects of FW obtained from colonic GI model digests of cvs. Amachi and Leona. Cytotoxicity was evaluated by the leakage of LDH in the culture media after 24 h treatment. LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but it is released from necrotic cells with damaged membranes [53]. When cancer cells are exposed to high concentrations of compounds with anticancer properties, elevation of LDH in the cell culture medium is well established as a marker of necrosis [54]. The concentrations of anthocyanins and anthocyanin metabolites corresponding to doses of FW used are shown in Table 2. The LDH values were expressed as percent change relative to the untreated control that contained medium without digest. No changes in cytotoxicity were observed following treatment with FW alone in either cell line (Figure 3A,B).

Table 2. Concentrations of total anthocyanins corresponding to the doses of FW used in cell culture viability experiments, calculated from the concentrations in V3, V4 and V5 in Table 1.

Dose	Anthocyanin Concentration (mg/L Cyanidin 3-Rutinoside Equivalents)	
% FW in Cell Culture Media	Cv. Amachi	Cv. Leona
0%	0	0
10%	0.203	0.091
25%	0.507	0.228
50%	1.013	0.457
75%	1.520	0.685
100%	2.027	0.913

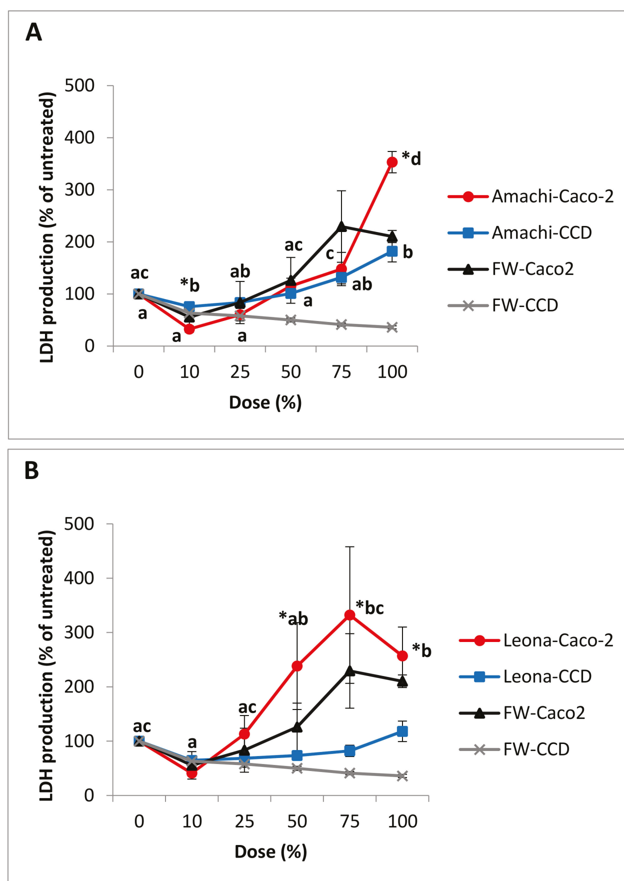


Figure 3. Effect of in vitro digests of cvs. Amachi (**A**) and Leona (**B**) on LDH production by Caco-2 and CCD-112CoN cells. Values are means ± SE of four independent experiments. Statistical analysis was performed via two-way ANOVA using cell line (Caco-2 versus CCD-112CoN) and dose (0%, 10%, 25%, 75% and 100%) as factors. Within each cell line, mean LDH values without a common letter are significantly different ($p < 0.05$). Between cell lines and within each dose, the symbol * represents a significant difference ($p < 0.05$) in the comparison between Caco-2 and CCD112-CoN cells at a specific digest dose.

In Caco-2 cells, the colonic FW digest of Amachi at the 100% dose was associated with a significant ($p < 0.05$) increase in LDH production by 253% relative to untreated controls while the 10% dose showed significantly ($p < 0.05$) lower LDH release (Figure 3A). In comparison to Amachi digests, the digests of Leona exhibited greater cytotoxicity as the 75% dose in addition to the 100% dose was associated with a significant ($p < 0.05$) rise in LDH production of 232% and 157% compared to control cells, respectively (Figure 3B). The MTT viability assay findings were in concordance with the above results as FW from Amachi and Leona elicited a dose response decrease in cell viability in Caco-2 cells (Figure 4). In comparison to unexposed controls, Amachi FW showed significantly lower cell viability at the 75% and 100% dose while FW from Leona treatment showed lower cell viability at the 50%, 75% and 100% concentrations. Caco-2 cells demonstrated a significantly ($p < 0.05$) higher susceptibility to the Leona FW with an IC_{50} value of 1.02 mg/L versus 2.66 mg/L for FW from Amachi. The Leona FW exhibited significantly ($p < 0.05$) greater decrease in viability at the 50% and 75% doses than Amachi FW (Figure 4). The above findings agree with studies showing that simulated gastric and small intestine digestion of the high anthocyanin-containing Vitelotte cultivar was associated with decreased viability of Caco-2 cells in a concentration-dependent manner [27]. A dose-related inhibition of Caco-2 cell proliferation was also observed following exposure to freeze-dried extracts of anthocyanin-rich colored potatoes [25]. The present findings extend these latter results to demonstrate that colonic microbial metabolites of anthocyanin-rich colored potato meals decrease Caco-2 colonic cell viability in a cultivar-dependent manner. In that regard, despite the greater antioxidant capacity and higher anthocyanin content associated with the Amachi colonic digests, greater cytotoxicity was observed with the colonic FW from Leona. Such findings might be related to differences in anthocyanin profiles in the FW digests, particularly the approximately two-fold greater content of the aglycone cyanidin and petunidin anthocyanins in the Leona versus Amachi colonic reactors. Cyanidin, but not its glycosides, was shown to be a potent inhibitor of the growth of human colon carcinoma HT-29 cells [55] or HCT-116 [56] and Caco-2 [57] colon cancer cells. Aglycones such as cyanidin can readily be released following intestinal microbial beta-glucosidase-mediated hydrolysis of anthocyanins [58]. Thus, the presence of cyanidin as an intermediate microbial metabolite could provide chemopreventative properties towards colorectal cancer. Relatively greater concentrations of petunidin anthocyanins were related to pro-apoptotic effects of extracts of purple-fleshed potatoes in human HCT-116 colon carcinoma cells while antioxidant capacity and chlorogenic acid content was unconnected to the cancer cell inhibition [59]. Possible mechanisms of cytotoxicity could be related to anthocyanins acting as pro-oxidants leading to the generation of cytotoxic reactive oxygen species, which has been associated with anthocyanin exposure with tumor cells [18]. Oxidative stress has been indicated to lead to cancer cell death via induction of mitochondrial caspase-dependent and caspase-independent pathways of apoptosis, which have been shown following anthocyanin exposure [60]. It is also conceivable that unmeasured potato components and microbial metabolites were involved in the differing cytotoxicity and cell viability outcomes.

Cultivar differences were also noted in terms of cytotoxic effects of the FW digests on the non-tumorigenic CCD-112CoN cells. The Amachi digests were associated with enhanced cytotoxicity in the non-tumorigenic CCD-112CoN cells, which showed a significant ($p < 0.05$) increase in LDH release by 82% versus untreated cells at the 100% dose (Figure 3A). It has been well described that cytotoxic agents that promote necrosis in cancer cells can also induce toxicity to normal cells at higher concentrations [61]. On the other hand, no significant increase in LDH release was noted in the CCD-112CoN cells exposed to cv. Leona FW. The Caco-2 cancer cell line was generally more sensitive than the CCD-112CoN cells to cytotoxicity associated with FW from both cultivars. There was significantly ($p < 0.05$) higher LDH response to cv. Amachi digests in Caco-2 cells in comparison to CCD-112CoN cells at the 100% dose. The cv. Leona digests elicited significantly ($p < 0.05$) higher LDH release by Caco-2 versus CCD-112CoN cells at doses of 50% (238% versus 73.5% of untreated cells), 75%, (332% versus 82% of untreated cells) and 100% (257% versus 118% of untreated cells), respectively. Relative differences between tumor and normal colonic cell lines have been previously

shown with anthocyanin-rich extracts, which demonstrated no significant toxicity towards normal colonic cells [62]. Such findings provide support for the concept that in vivo colonic exposure to digests of anthocyanin-rich potatoes can damage cancer cells with limited adverse effects on normal colonic cells. Further research is needed to measure the small molecular weight molecules in the colonic digests that could be tested on different tumorigenic and non-tumorigenic cell lines to better clarify the anticancer activities of the metabolites.

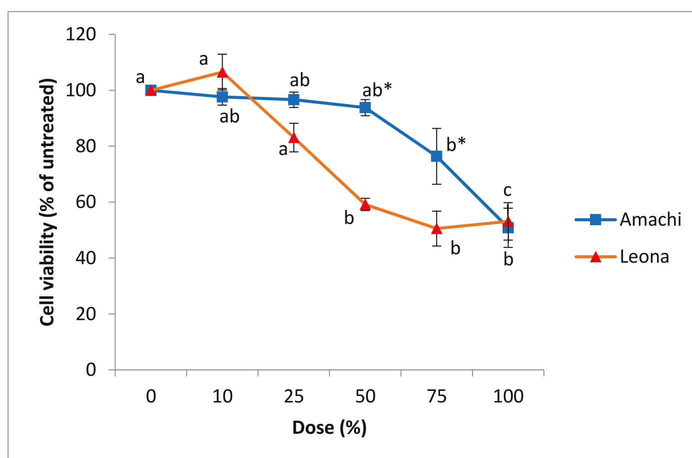


Figure 4. Effect of in vitro digests of cvs. Amachi and Leona on the viability of Caco-2 cells as assessed via the the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Values are means ± SE of four independent experiments. Means within cultivar treatment without a common letter are significantly different ($p < 0.05$). The symbol * represents the comparison between cvs. Amachi and Leona at a specific digest dose as significantly different ($p < 0.05$).

4. Conclusions

To our knowledge, this is the first study to report the effects of human simulated dynamic gastrointestinal digestion on anthocyanin profiles and antioxidant capacity in pigmented potatoes, as well as the effects of their FW digests on tumor and non-tumorigenic cell cytotoxicity and viability. Overall, the results of the present study revealed that the genetic background of the two purple potato cultivars analyzed led to major variances in composition and total content of anthocyanins throughout the GI model. An increase in antioxidant capacity with digestion time in all vessels was noted with both cultivars. This latter finding was likely due to antioxidant properties of the parent anthocyanin compounds in the upper intestinal vessels, whereas smaller phenolics could have contributed to the antioxidant effects in the colonic reactors. In our study, microbial metabolism can account for the anthocyanin degradation as seen by diminished concentrations of several anthocyanin species in the colonic vessels, which was particularly evident in the descending V5 colonic vessel of the Amachi cv. Our GI digestion model studies concurrently illustrate an increase in bioaccessibility associated with microbial biotransformation as shown by the initial appearance of several anthocyanin compounds throughout the colonic vessels in both tested potato cultivars. There was a greater than seven-fold higher concentrations of anthocyanins in the descending V5 colonic reactor of Leona versus Amachi, despite the several-fold higher total anthocyanin content in the overall GI model for the Amachi cv. The physiological relevance of this latter finding requires more investigation.

Interestingly, Leona FW showed relatively more potent effects on cytotoxicity and cell viability on colonic tumor cells. Unlike Amachi FW, the FW from Leona did not exhibit adverse effects on normal colonic cells. Further studies are needed to determine the identification of the bioactive components in

the digesta and their mechanisms of action of cytotoxicity. The types of anthocyanins that contribute to the health promoting effects of purple potatoes require more research, including the bioavailability of anthocyanin metabolites. The examination of the digested anthocyanins and their metabolites generated from the gut model could be coupled with a cellular absorption model to assess for their bioavailabilities, which is presently in progress. This approach could help to identify the nature of bioactive anthocyanin compounds associated with the anti-cancer properties and other health attributes associated with the intake of purple potatoes. For example, antioxidant and antihypertensive effects of microwaved purple potatoes have been observed in hypertensive human subjects [63]. Insights arising from the above research can lead to objectives for potato breeding programs towards enhancing specific anthocyanins to develop varieties with chemopreventative properties.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/9/953/s1, Figure S1: Time course of chlorogenic acid measurement of digesta from gut model vessels following provision of a meal containing different potato meals.

Acknowledgments: The research was supported by CGIAR Research Program on Agriculture for Nutrition & Health (CRP-A4NH) and a Discovery Grant from the Natural Sciences and Engineering Council of Canada. We thank Severin Polreich (CIP) and Ladislao Palomino (Instituto Nacional de Innovación Agraria, INIA, Peru) for providing the Leona and Amachi potato tuber material used for this research.

Author Contributions: S.K. designed the study, oversaw all methodological and implementation aspects and wrote the manuscript. M.M.I. oversaw the cell culture experiments, and assisted in writing the manuscript, data interpretation, statistical analysis and graphical representations. E.M.-B. analyzed and interpreted the ESI-TOF-MS data. L.S. oversaw the ESI-TOF-MS measurements and analyses. K.S. performed the ESI-TOF-MS measurements. B.A. performed the in vitro digestions. E.H. performed the LDH and viability assays. S.P. oversaw the in vitro digestions. G.B. recommended the varieties used in this study, was responsible for sample preparation and provided guidance and input in all stages of the study including manuscript preparation. T.z.-F. led the overall project under which this study was conceived, worked on establishing the collaboration between McGill and CIP, arranged A4NH funding for supporting this study and provided input in all stages of the study including manuscript preparation.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Prior, R.L.; Wu, X. Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic. Res.* **2006**, *40*, 1014–1028. [CrossRef] [PubMed]
2. Takeoka, G.; Dao, L. *Methods of Analysis for Functional Foods and Nutraceuticals*, 1st ed.; Hurst, W.J., Ed.; CRC Press LLC: Boca Raton, FL, USA, 2002.
3. Brown, C.R.; Wrolstad, R.; Durst, C.-P.; Yang, B.A. Clevidence, Breeding studies in potatoes containing high concentrations of anthocyanins. *Am. J. Potato Res.* **2003**, *80*, 241–250. [CrossRef]
4. Ieri, F.; Innocenti, M.; Andrenelli, L.; Vecchio, V.; Mulinacci, N. Rapid HPLC/DAD/MS method to determine phenolic acids, glycoalkaloids and anthocyanins in pigmented potatoes (*Solanum tuberosum* L.) and correlations with variety and geographical origin. *Food Chem.* **2011**, *125*, 750–759. [CrossRef]
5. Bellumori, M.; Innocenti, M.; Michelozzi, M.; Cerratini, L.; Mulinacci, N. Coloured-fleshed potatoes after boiling: Promising sources of known antioxidant compounds. *J. Food Compos. Anal.* **2017**, *59*, 1–7. [CrossRef]
6. Rechner, A.R.; Smith, M.A.; Kuhnle, G.; Gibson, G.R.; Debnam, E.S.; Srai, S.K.S.; Moore, P.K.; Rice-Evans, C.A. Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products. *Free Radic. Biol. Med.* **2004**, *36*, 212–225. [CrossRef] [PubMed]
7. McDougall, G.J.; Dobson, P.; Smith, P.; Blake, A.; Stewart, D. Assessing potential bioavailability of raspberry anthocyanins using an In Vitro digestion system. *J. Agric. Food Chem.* **2005**, *53*, 5896–5904. [CrossRef] [PubMed]
8. Podsędek, A.; Redzynia, M.; Klewicka, E.; Koziołkiewicz, M. Matrix effects on the stability and antioxidant activity of red cabbage anthocyanins under simulated gastrointestinal digestion. *Biomed. Res. Int.* **2014**. [CrossRef] [PubMed]
9. Blanquet-Diot, S.; Deat, E.; Jarrige, J.F.; Denis, S.; Beyssac, E.; Alric, M. Combining the dynamic TNO-gastrointestinal tract system with a Caco-2 cell culture model: Application to the assessment of lycopene and alpha-tocopherol bioavailability from a whole food. *J. Agric. Food Chem.* **2009**, *57*, 11314–11320.

10. Martoni, C.; Bhathena, J.; Jones, M.L.; Urbanska, A.M.; Chen, H.; Prakash, S. Investigation of microencapsulated BSH active Lactobacillus in the simulated human GI tract. *J. Biomed. Biotechnol.* **2007**, *7*, 1–9. [CrossRef] [PubMed]
11. Molly, K.; Vande Woestyne, M.; De Smet, I.; Verstraete, W. Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. *Microb. Ecol. Health Dis.* **1994**, *7*, 191–200. [CrossRef]
12. Liang, L.; Wu, X.; Zhao, T.; Zhao, J.; Li, F.; Zou, Y.; Mao, G.; Yang, L. In Vitro bioaccessibility and antioxidant activity of anthocyanins from mulberry (*Morus atropurpurea* Roxb.) following simulated gastro-intestinal digestion. *Food Res. Int.* **2012**, *46*, 76–82.
13. Noguer, M.; Cerezo, A.B.; Rentzsch, M.; Winterhalter, P.; Troncoso, A.M.; García-Parrilla, M.C. Simulated digestion and antioxidant activity of red wine fractions separated by high speed countercurrent chromatography. *J. Agric. Food Chem.* **2008**, *56*, 8879–8884. [CrossRef] [PubMed]
14. Srivastava, A.; Akoh, C.C.; Fischer, J.; Krewer, G. Effect of anthocyanin fractions from selected cultivars of Georgia-grown blueberries on apoptosis and phase II enzymes. *J. Agric. Food Chem.* **2007**, *55*, 3180–3185. [CrossRef] [PubMed]
15. Katsube, N.; Iwashita, K.; Tsushida, T.; Yamaki, K.; Kobori, M. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *J. Agric. Food Chem.* **2003**, *51*, 68–75. [CrossRef] [PubMed]
16. Bornsek, S.M.; Ziberna, L.; Polak, T.; Vanzo, A.; Ulrich, N.P.; Abram, V.; Tramer, F.; Passamonti, S. Bilberry and blueberry anthocyanins act as powerful intracellular antioxidants in mammalian cells. *Food Chem.* **2012**, *134*, 1878–1884. [CrossRef] [PubMed]
17. Dai, J.; Gupte, A.; Gates, L.; Mumper, R.J. A comprehensive study of anthocyanin-containing extracts from selected blackberry cultivars: Extraction methods, stability, anticancer properties and mechanisms. *Food Chem. Toxicol.* **2009**, *47*, 837–847. [CrossRef] [PubMed]
18. Rugina, D.; Sconta, Z.; Leopold, L.; Pintea, A.; Bunea, A.; Socaciu, C. Antioxidant activities of chokeberry extracts and the cytotoxic action of their anthocyanin fraction on HeLa human cervical tumor cells. *J. Med. Food* **2012**, *15*, 700–706. [CrossRef] [PubMed]
19. Charepalli, V.; Reddivari, L.; Vadde, R.; Walia, S.; Radhakrishnan, S.; Vanamala, J.K.P. Eugenia jambolana (*Java plum*) fruit extract exhibits anti-cancer activity against early stage human HCT-116 colon cancer cells and colon cancer stem cells. *Cancers* **2016**, *8*, 29. [CrossRef] [PubMed]
20. Lim, S.; Xu, J.; Kim, J.; Chen, T.-Y.; Su, X.; Standard, J.; Edward, C.; Jason, G.; Betty, H.; Katz, B.; et al. Role of anthocyanin-enriched purple-fleshed sweet potato p40 in colorectal cancer prevention. *Mol. Nutr. Food Res.* **2013**, *57*, 1908–1917. [CrossRef] [PubMed]
21. Kang, S.Y.; Seeram, N.P.; Nair, M.G.; Bourquin, L.D. Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. *Cancer Lett.* **2003**, *194*, 13–19. [CrossRef]
22. Bobe, G.; Want, B.; Seeram, N.P.; Nair, M.G.; Bourquin, L.D. Dietary anthocyanin-rich tart cherry extract inhibits intestinal tumorigenesis in APC(Min) mice fed suboptimal levels of sulindac. *J. Agric. Food Chem.* **2006**, *54*, 9322–9328. [CrossRef] [PubMed]
23. Harris, G.K.; Gupta, A.; Nines, R.G.; Kresty, L.A.; Habib, S.G.; Frankel, W.L.; LaPerle, K.; Gallaher, D.D.; Schwartz, S.J.; Stoner, G.D. Effects of lyophilized black raspberries on azoxymethane-induced colon cancer and 8-hydroxy-2′-deoxyguanosine levels in the Fischer 344 rat. *Nutr. Cancer* **2001**, *40*, 125–133. [CrossRef] [PubMed]
24. Hagiwara, A.; Miyashita, K.; Nakanishi, T.; Sano, M.; Tamano, S.; Kadota, T.; Koda, T.; Nakamura, M.; Imaida, K.; Ito, N.; et al. Pronounced inhibition by a natural anthocyanin, purple corn color, of 2-amino-l-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-associated colorectal carcinogenesis in male F344 rats pretreated with 1,2-dimethylhydrazine. *Cancer Lett.* **2001**, *171*, 17–25. [CrossRef]
25. Wang, Q.; Chen, Q.; He, M.; Mir, P.; Su, J.; Yang, Q. Inhibitory effect of antioxidant extracts from various potatoes on the proliferation of human colon and liver cancer cells. *Nutr. Cancer* **2011**, *53*, 1044–1052. [CrossRef] [PubMed]
26. Madiwale, G.P.; Reddivari, L.; Stone, M.; Holm, D.G.; Vanamala, J. Combined effects of storage and processing on the bioactive compounds and pro-apoptotic properties of color-fleshed potatoes in human colon cancer cells. *J. Agric. Food Chem.* **2012**, *60*, 11088–11096. [CrossRef] [PubMed]

27. Ombra, M.N.; Fratianni, F.; Granese, T.; Cardinale, F.; Cozzolino, A.; Nazzaro, F. In Vitro antioxidant, antimicrobial and anti-proliferative activities of purple potato extracts (*Solanum tuberosum* cv. *Vitelotte noire*) following simulated gastro-intestinal digestion. *Nat. Prod. Res.* **2014**, *29*, 1087–1091. [PubMed]
28. Fonseca, C.; Burgos, G.; Rodríguez, F.; Muñoa, L.; Ordinola, M. *Catálogo De Variedades De Papa Nativa Con Potencial Para La Seguridad Alimentaria Y Nutricional De Apurímac Y Huancavelica*; Centro Internacional De La Papa: Lima, Peru, 2014.
29. Porras, E.; Burgos, G.; Sosa, P.; Zum Felde, T. *Procedures for Sampling and Sample Preparation of Sweetpotato Roots and Potato Tubers for Mineral Analysis*; International Potato Center (CIP), Global Program Genetics and Crop Improvement: Lima, Peru, 2014; p. 13. ISBN 978-92-9060-445-7.
30. Feldman, M.; Cryer, B.; McArthur, K.E.; Huet, B.A.; Lee, E. Effects of aging and gastritis on gastric acid and pepsin secretion in humans: A prospective study. *Gastroenterology* **1996**, *110*, 1043–1052. [CrossRef] [PubMed]
31. Tian, Q.; Konczak, I.; Schwartz, S. Probing anthocyanin profiles in purple sweet potato cell line (*Ipomea batatas* L. cv. Ayamurasaki) by high performance liquid chromatography and electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 6503–6509. [CrossRef] [PubMed]
32. Benzie, I.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef] [PubMed]
33. Teng, C.P.; Mya, K.Y.; Win, K.Y.; Yeo, C.C.; Low, M.; He, C.; Han, M.-Y. Star-shaped polyhedral oligomeric silsesquioxane-polycaprolactone-polyurethane as biomaterials for tissue engineering application. *NPG Asia Mater.* **2014**, *6*, e142. [CrossRef]
34. Vanden Berghe, T.; Grootjans, S.; Goossens, V.; Dondelinger, Y.; Krysko, D.V.; Takahashi, N.; Vandenabeele, P. Determination of apoptotic and necrotic cell death In Vitro and In Vivo. *Methods* **2013**, *61*, 117–129. [CrossRef] [PubMed]
35. Mossman, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]
36. Andre, C.M.; Oufir, M.; Guignard, C.; Hoffman, L.; Hausman, J.-F.; Evers, D.; Larondelle, Y. Antioxidant profiling of native Andean potato tubers (*Solanum tuberosum* L.) reveals cultivars with high levels of β-carotene, α-tocopherol, chlorogenic acid, petanin. *J. Agric. Food Chem.* **2007**, *55*, 10839–10849. [CrossRef] [PubMed]
37. Giusti, M.M.; Polit, M.F.; Ayvaz, H.; Tay, D.; Manrique, I. Characterization and quantitation of anthocyanins and other phenolics in native Andean potatoes. *J. Agric. Food Chem.* **2014**, *62*, 4408–4416. [CrossRef] [PubMed]
38. Eichhorn, S.; Winterhalter, P. Anthocyanins from pigmented potato (*Solanum tuberosum* L.) varieties. *Food Res. Int.* **2005**, *38*, 943–948. [CrossRef]
39. Kim, H.J.; Kim, H.M.; Lee, K.G.; Shin, J.S.; Ahn, H.J.; Jeong, J.C.; Kwon, O.K.; Nam, J.H.; Lee, K.T.; Jang, D.S. *p*-Coumaroyl Anthocyanins from the Tuber Epidermis of a Colored Potato *Solanum tuberosum* L. cv Jayoung. *Bull. Korean Chem. Soc.* **2014**, *35*, 8. [CrossRef]
40. Lewis, C.; Walker, J.; Lancaster, J.; Sutton, K. Determination of anthocyanins, flavonoids and phenolic acids in potatoes. I: Colored Cultivars of *Solanum tuberosum* L. *J. Sci. Food Agric.* **1998**, *77*, 45–57.
41. Aura, A.-M.; Martin-Lopez, P.; O'Leary, K.A.; Williamson, G.; Oksman-Caldentey, K.-M.; Poutanen, K.; Santos-Buelga, C. In Vitro metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* **2005**, *44*, 133–142. [CrossRef] [PubMed]
42. Kubow, S.; Iskandar, M.M.; Sabally, K.; Azadi, B.; Sadeghi Ekbatan, S.; Kumarathasan, P.; Dhar Das, D.; Prakash, S.; Burgos, G.; Zum Felde, T. Biotransformation of anthocyanins from two purple-fleshed sweet potatoo accessions in a dynamic gastrointestinal system. *Food Chem.* **2016**, *192*, 171–177. [CrossRef] [PubMed]
43. Woodward, G.; Kroon, P.; Cassidy, A.; Colin, K. Anthocyanin stability and recovery: Implications for the analysis of clinical and experimental samples. *J. Agric. Food Chem.* **2009**, *57*, 5271–5278. [CrossRef] [PubMed]
44. Fang, J. Some anthocyanins could be efficiently absorbed across the gastrointestinal mucosa: Extensive presystemic metabolism reduces apparent bioavailability. *J. Agric. Food Chem.* **2014**, *62*, 3904–3911. [CrossRef] [PubMed]
45. Perez-Vicente, A.; Gil-Izquierdo, A.; Garcia-Viguera, C. In Vitro gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *J. Agric. Food Chem.* **2002**, *50*, 2308–2312. [CrossRef] [PubMed]

46. Kamiloglu, S.; Capanoglu, E. Investigating the In Vitro bioaccessibility of polyphenols in fresh and sun-dried figs (*Ficus carica* L.). *Int. J. Food Sci.* **2013**, *48*, 2621–2629. [CrossRef]
47. Bouayed, J.; Hoffmann, L.; Bohn, T. Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal digestion and dialysis of apple varieties: Bioaccessibility and potential uptake. *Food Chem.* **2011**, *128*, 14–21. [CrossRef] [PubMed]
48. Flores, F.P.; Singh, R.K.; Kerr, W.L.; Phillips, D.R.; Kong, F. In Vitro release properties of encapsulated blueberry (*Vaccinium ashei*) extracts. *Food Chem.* **2015**, *168*, 225–232. [CrossRef] [PubMed]
49. Gálvez, J.; Coelho, G.; Crespo, M.E.; Cruz, T.; Rodríguez-Cabezas, M.E.; Concha, A.; Gonzalez, M.; Zarzuelo, A. Intestinal anti-inflammatory activity of morin on chronic experimental colitis in the rat. *Aliment. Pharmacol. Ther.* **2001**, *15*, 2027–2039. [CrossRef] [PubMed]
50. Lala, G.; Malik, M.; Zhao, C.; He, J.; Kwon, Y.; Giusti, M.M.; Magnuson, B.A. Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats. *Nutr. Cancer* **2006**, *54*, 84–93. [CrossRef] [PubMed]
51. Garsetti, M.; Pellegrini, N.; Baggio, C.; Brighenti, F. Antioxidant activity in human faeces. *Br. J. Nutr.* **2000**, *84*, 705–710. [PubMed]
52. Cerdá, B.; Espín, J.C.; Parra, S.; Martínez, P.; Tomás-Barberán, F.A. The potent In Vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy–6H–dibenzopyran–6–one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43*, 205–220. [CrossRef] [PubMed]
53. Chengbin, X.; Wei, L.; Jianhong, W.; Xiangliang, Y.; Huibi, X. Chemoprotective effect of *N*-acetylcysteine (NAC) on cellular oxidative damages and apoptosis induced by nano titanium dioxide under UVA irradiation. *Toxicol. In Vitro* **2011**, *25*, 110–216.
54. Lin, W.S.; Huang, Y.W.; Zhou, X.D.; Ma, Y.F. In Vitro toxicity of silica nanoparticles in human lung cancer cells. *Toxicol. Appl. Pharmacol.* **2006**, *217*, 252–259. [CrossRef] [PubMed]
55. Briviba, K.S.; Abrahamse, L.; Pool-Zobel, B.L.; Rechkemmer, G. Neurotensin- and EGF-induced metabolic activation of colon carcinoma cells is diminished by dietary flavonoid cyanidin but not by its glycosides. *Nutr. Cancer* **2001**, *41*, 172–179. [CrossRef] [PubMed]
56. Zhang, Y.J.; Vareed, S.D.; Nair, M.G. Human tumor cell growth inhibition by nontoxic anthocyanidins in fruits and vegetables. *Life Sci.* **2005**, *76*, 1465–1472. [CrossRef] [PubMed]
57. Takeuchi, M.; Ohtani, K.; Ma, Y.; Kato, S.; Semba, S.; Katoh, T.; Wakamiya, N.; Taniguchi, T. Differential effects of cyanidin and cyanidin-3-glucoside on human cell lines. *Food Sci. Technol. Res.* **2011**, *17*, 515–521. [CrossRef]
58. Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi, S.W.; Kawakishi, S.; Osawa, T. Antioxidative activity of the anthocyanin pigments, cyanidin-3-O-h-D-glucoside and cyanidin. *J. Agric. Food Chem.* **1994**, *42*, 2407–2410. [CrossRef]
59. Stushnoff, C.; Holm, D.; Thompson, M.D.; Jiang, W.; Thompson, H.J.; Joyce, N.I.; Wilson, P. Antioxidant properties of cultivars and selections from the Colorado potato breeding program. *Am. J. Potato Res.* **2008**, *85*, 267–276. [CrossRef]
60. Lin, B.-W.; Gong, C.-C.; Song, H.-F.; Cui, Y.-Y. Effects of anthocyanins on the prevention and treatment of cancer. *Br. J. Pharmacol.* **2017**, *174*, 1226–1243. [CrossRef] [PubMed]
61. Formigli, L.; Papucci, L.; Tani, A.; Schiavone, N.; Tempestini, A.; Orlandini, G.E.; Capaccioli, S.; Orlandini, S.Z. Aponecrosis: Morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. *J. Cell. Physiol.* **2000**, *182*, 41–49. [CrossRef]
62. Zhao, C.; Giusti, M.M.; Malik, M.; Moyer, M.P.; Magnuson, B. Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *J. Agric. Food Chem.* **2004**, *52*, 6122–6128. [CrossRef] [PubMed]
63. Vinson, J.A.; Demkosky, C.A.; Navarre, D.A.; Smyda, M.A. High-antioxidant potatoes: Acute in vivo antioxidant source and hypotensive agent in humans after supplementation to hypertensive subjects. *J. Agric. Food Chem.* **2012**, *60*, 6749–6754. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Flavonoid-Rich Extract of *Paulownia fortunei* Flowers Attenuates Diet-Induced Hyperlipidemia, Hepatic Steatosis and Insulin Resistance in Obesity Mice by AMPK Pathway

Chanmin Liu [1,*], Jieqiong Ma [2], Jianmei Sun [1], Chao Cheng [1], Zhaojun Feng [1], Hong Jiang [1] and Wei Yang [1]

[1] School of Life Science, Jiangsu Normal University, No. 101, Shanghai Road, Tangshan New Area, Xuzhou 221116, Jiangsu, China; sunjianmei@jsnu.edu.cn (J.S.); chengchao@jsnu.edu.cn (C.C.); fzj@jsnu.edu.cn (Z.F.); jhxznu@126.com (H.J.); 15852113162@163.com (W.Y.)

[2] School of Chemical Engineering, Sichuan University of Science and Engineering, No. 180, Huixing Road, Zigong 643000, Sichuan, China; jieqingma@126.com

* Correspondence: chanminliu@126.com; Tel./Fax: +86-0516-8340-3170

Received: 19 June 2017; Accepted: 28 August 2017; Published: 30 August 2017

Abstract: The flavonoid-rich extract from *Paulownia fortunei* flowers (EPF) has been reported to prevent obesity and other lipid metabolism disease. However, the mechanism of its protective effects is not yet clear. The objective of this study was to investigate molecular factors involved in the hypoglycemic and hypolipidemic effects of EPF in obese mice fed a high-fat diet (HFD). Male h ICR (Institute of Cancer Research) mice were fed a HFD containing or not containing the EPF (50 or 100 mg/kg) for eight weeks. EPF reduced body weight gain, lipid accumulation in livers and levels of lipid, glucose and insulin in plasma as well as reduced insulin resistance as compared with the HFD group. EPF significantly decreased serum aminotransferase activity of the HFD group. We observed that EPF administration significantly increased the level of AMP-activated kinase (AMPK) phosphorylation and prevented fat deposits in livers and HepG2 cells, but these effects were blocked by compound C (an AMPK inhibitor). The protective effects of EPF were probably associated with the decrease in HMGCR, SREBP-1c and FAS expressions and the increase in CPT1 and phosphor-IRS-1 expressions. Our results suggest that EPF might be a potential natural candidate for the treatment and/or prevention of overweight and hepatic and metabolic-related alterations induced by HFD.

Keywords: *Paulownia fortunei* flower; hyperlipidemia; hyperglycemia; obesity; hepatic fat accumulation; lipid metabolism; AMPK

---

1. Introduction

High fat diet (HFD) could induce hepatic steatosis, hyperlipidemia, obesity, diabetes and other lipid metabolism disorder disease by regulating signal pathway of lipid metabolism in livers [1,2]. AMP-activated kinase (AMPK), a phylogenetically conserved serine/threonine protein kinase, is a key regulator of energy metabolic homeostasis and a crucial target for drugs both ancient and modern [3]. Activation of AMPK can play an important role in regulating energy balance and nutrient metabolism, such as the synthesis of fatty acids, cholesterol, glucose, and hepatic gluconeogenesis and translation. Moreover, AMPK is considered an attractive target for the prevention of diseases such as obesity, diabetes, inflammation and cancer [3,4]. It is reported that AMPK could be activated by natural phenolic compound, such as resveratrol, epigallocatechin gallate (EGCG), curcumin, quercetin, caffeic acid phenethyl ester (CAPE), berberine, and theaflavin [4].

Indigenous medicinal plants have been recommended for treatment of many metabolism diseases because of their easy availability and relatively fewer side effects [1,3,5]. Epidemiological studies revealed that an increased daily intake of phenolic compounds in dietary supplements act as anti-oxidant and anti-inflammatory agents to increase thermogenesis and energy expenditure while decreasing inflammation and oxidative stress, further supporting progress towards decreased metabolic disorders [1,5]. Previous studies confirmed that the species of the genus *Paulownia* (belonging to the family scrophulariaceae) exhibited a broad spectrum of biological effects, including antioxidant, anticarcinogenic [6,7], antiphlogistic [8], antiviral [9], antimicrobial [10] and anti-cholinesterase activities [11]. *Paulownia fortunei* (Seem.) Hemsl. is a fast growing ornamental tree, native to Mainland China and distributed almost all over the world [10]. This species is renowned as a polyphenol rich plant, which has been used in traditional Chinese medicine for the treatment of hypertension, enteritis, tonsillitis, bronchitis and dysentery [7,12,13]. The flowers of *P. fortunei* are also used as an additive to seasonal foods in China, made into a local delicacy called "Zheng Cai". Previous studies have shown that flower extracts of *P. fortunei* (EPF) contain high amounts of flavonoids, which are mainly composed of apigenin, luteolin, hesperetin, β-sitosterol, thunberginol A, daucosterol, quercetin, kaempferol, and their derivatives [12,13]. In previous study, we showed that flavonoid puerarin could prevent hyperlipidemia, oxidative stress and other lipid metabolism disorder disease in livers [14,15]. For these reasons, we hypothesized that the extract from *P. fortunei* flowers (EPF) might play an important role in protecting HFD-induced hyperlipidemia, insulin resistance and hepatic fat accumulation. In this study, for the first time, we used high-fat diet induced obese mice to verify the protective effects of ethanol extract of *P. fortunei* flowers containing the flavonoids and to study the mechanisms focusing on these effects in enhancing AMPK signaling pathway and inhibiting lipid metabolism disorder in livers.

2. Materials and Methods

2.1. Chemical Reagents

Antibodies against HMGCR (sc-27578), pMAPK (T172) (CST-2535), AMPK (CST-2532), pIRS-1 (Ser-307) (sc-101709), IRS-1 (sc-51517), FAS (sc-715), CPT1 (CST-12252), SREBP-1c (sc-13551) and β-actin (sc-1616) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology (Beverly, MA, USA). Rutin, luteolin, and apigenin (>99%) were obtained from Sigma Chemical Co. (Saint Louis, MO, USA). Quercetin 3-*O*-glucoside, luteolin 7-*O*-glucoside, kaempferol 3-*O*-glucoside, and apigenin 7-*O*-glucoside (>99%) were purchased from Shanghai Yongye Biotechnology Co., Ltd. or Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). All other solvents and reagents were purchased from Aladdin (Aladdin, Shanghai, China).

2.2. Sample Preparation

The flowers of *P. fortunei* were collected at Xuzhou (Jiangsu Province, China) in April 2016. Eight kilograms of dried flowers were pulverized into powders and extracted three times with 70% ethanol (5 L) at 40 °C for 48 h. After filtration and removal of residues, the extract was submitted to a spray-drying (180 °C inlet temperature and 100 °C outlet temperature). The isolated flavonoids were purified by Sephadex LH-20 column chromatography. Total flavonoid content was determined according to Samad et al. by the following colorimetric method, where catechin was used as a standard. Catechin concentrations ranging from 0.05 to 0.5 mg/mL were used to generate the standard calibration curve [16]. The product yield is 5.28%. The flower extracts of *P. fortunei* (EPF) contain high amounts of flavonoids (67.8% in EPF).

The content (mg/g dry weight) of major flavonoids was determined by high performance liquid chromatography (HPLC) according to the method described with slight modifications [17,18] (Table 1). Briefly, HPLC analyses were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) consisting in a binary pump, an autosampler and a diode-array detector (DAD). A ZORBAX SB-C18 (4.6 × 150 mm, 5 μm particle diameter, Agilent Technologies,

Santa Clara, CA, USA) column was employed. Gradient elution was performed with solution A, composed of 50 mM sodium phosphate (pH 3.3) and 10% methanol, and solution B, comprising 70% methanol, delivered at a flow rate of 1.0 mL/min as follows: initially 100% of solution A; for the next 15 min, 70% A; for another 30 min, 65% A; for another 20 min, 60% A; for another 5 min, 50% A; and finally 0% A for 25 min. The injection volume for the extract was 10 μL. A library was made, comprising retention times on HPLC and spectra of standard chemical compounds. The extracts were then analyzed using the same HPLC system. The detected polyphenol peaks were compared with respect to retention time with those in the library.

Table 1. Content of major flavonoid compounds in the flower extracts of *P. fortunei* (EPF) used in the study.

Compound	Content (mg/g Dry Weight)
Apigenin	16.63 ± 0.01
Luteolin	7.16 ± 0.04
Rutin	1.87 ± 0.02
Luteolin 7-*O*-glucoside	9.26 ± 0.02
Kaempferol 3-*O*-glucoside	5.17 ± 0.01
Apigenin 7-*O*-glucoside	4.13 ± 0.03
Quercetin 3-*O*-glucoside	3.95 ± 0.04

Data are expressed as mean ± standard error (SE) (n = 3). Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test.

2.3. Cell Culture and Treatments

Human HepG2 hepatocytes were cultured and treated previously described [19]. Briefly, human hepatoma-derived HepG2 cells were maintained in low glucose-containing Dulbecco's modied Eagle's medium. After reaching 75% confluence, the cells were serum-starved for 16 h and then exposed to FFA (free fatty acids) to induce fat overloading. The cells were treated with EPF (10–100 g/mL) or FFA (0.25–2 mM). Cellular triglyceride (TG) was detected with related kits as manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Animals and Experimental Procedure

The present research reported in this paper was conducted in accordance with the Chinese legislation and NIH publications on the use and care of laboratory animals. Relevant university committees for animal experiments approved these experiments, ethic approval number: GB14925-2001; JSNUCAE-2017-12.

Male ICR mice (20–25 g) were purchased from the Branch of National Breeder Center of Rodents (Beijing, China). Mice were maintained in an environmentally controlled room (23 ± 2 °C, 55 ± 10% humidity) for 1 week for acclimatization. Then, fifty mice were randomly assigned to five groups (10 mice/group).

Group I (vehicle control) mice were fed a standard normal chow diet (SND) consisting of 60% kcal carbohydrate, 24% kcal protein, and 16% kcal fat, with a total energy of 3.1 kcal/g. Mice in Group II, Group III and Group IV were fed a high fat diet (HFD) consisting of 21.3% kcal carbohydrate, 18.4% kcal protein, and 60.3% kcal fat, with a total energy of 5.1 kcal/g. Mice in Group III and Group IV were fed a high fat diet (HFD) and daily given EPF in distilled water containing 0.1% Tween 80 at two doses 50 and 100 mg/(kg day), respectively. Mice in Group V (EPF 100 mg/kg) were fed a standard normal chow diet (SND) and received EPF in distilled water containing 0.1% Tween 80 at a dose of 100 mg/(kg day) by oral gavage. The EPF concentration was set to the maximum concentration that had not affected the food intake in a preliminary experiment [20]. The food intake and body weight of the animals were measured daily.

The experiment lasted for eight weeks. At the end of treatment, mice were sacrificed and blood samples were drawn by cardiac puncture with heparined tubes. The plasma was separated by

centrifugation (3000× *g* for 10 min at 4 °C) and stored at −70 °C until analysis. The liver tissues was immediately excised for experiments or stored at −70 °C for later use.

2.5. Biochemical Analysis

The levels of serum glucose, total cholesterol (TC), triglyceride (TG), density lipoprotein (LDL), high-density lipoprotein (HDL), urea and creatinine and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [14,15].

2.6. Oral Glucose Tolerance

Oral glucose tolerance was determined as described previously [21]. Insulin levels were characterized by a corresponding mouse ELISA kit according to the manufacturer's instructions. Insulin resistance (IR) and the homeostatic index of insulin resistance (HOMA-IR) were determined as described previously (KingMed Diagnostics, Gongzhou China) [21].

2.7. Oil Red O Staining

Oil Red O was used to stain intracellular lipids as described previously [19]. Briefly, HepG2 cells in different groups were cultured in the corresponding medium for 24 h. Cells were then fixed with 4% paraformaldehyde and stained with a freshly prepared working solution of oil red O at room temperature. The histological changes of liver were evaluated using a commercial kit, stained by the Oil Red O solution (0.5%; Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol.

2.8. Measurement of Liver Triglyceride Content

Liver TG content was determined as described previously [14,22]. The levels of hepatic TG in the extraction solution were determined by enzymatic methods using commercially available kits (Elabscience Biotechnology, Wuhan, Hubei, China) according to the instructions of the manufacturer.

2.9. Western Blot Analyses

To measure the effect of EPF on gene expression in mouse livers, Western blot analysis was performed as previously described by us [14,15]. Total protein content was determined by BCA protein assay (Thermo Scientific Pierce, Rockford, IL, USA).

2.10. Statistical Analysis

Results were expressed as mean ± standard error (SE). Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test (ANOVA; $p < 0.05$).

3. Results

3.1. Identification of Purified EPF

We find that flower extracts of *P. fortunei* (EPF) contain high amounts of flavonoids (67.8% in EPF). As shown in Table 1, using standard chemical compound of flavonoids, seven kinds of flavonoids were identified in the extract from *P. fortunei* flowers. Apigenin and luteolin 7-*O*-glucoside were major flavonoids in the extract of *P. fortunei* flowers.

3.2. General Characteristics

Table 2 shows that mice fed HFD gained more body weight as compared with the control group ($p < 0.05$). The average daily food intake per mouse was greater in Group I (control) than that in Group II (HFD group). Since the calorie density of standard normal chow diet is less than that of high fat diet,

the average daily calorie intake per mouse was markedly less in the control group as compared with the HFD group ($p < 0.05$). However, treatment with EPF 50 and 100 mg/kg significantly ameliorated HFD-induced additional weight gain and calorie intake. The standard normal chow diet supplemented with EPF caused a slight decrease in body weight compared to the standard normal chow diet only, but this decline did not show a significant difference.

Table 2. Effect of EPF on the body weight gain, food intake and calorie intake.

Parameter	SND	HFD	HFD + EPF (50 mg/kg)	HFD + EPF (100 mg/kg)	SND + EPF (100 mg/kg)
Body weight gain (g)	10.26 ± 1.93^{a}	17.85 ± 3.12^{b}	13.67 ± 3.04^{c}	12.27 ± 2.71^{c}	10.09 ± 2.18^{a}
Food intake (g/day)	4.82 ± 0.15^{a}	3.93 ± 0.22^{b}	3.41 ± 0.13^{c}	3.42 ± 0.29^{c}	4.79 ± 0.24^{a}
Calorie intake (kcal/g/day)	14.94 ± 0.47^{a}	20.04 ± 1.12^{b}	17.39 ± 0.66^{c}	17.44 ± 1.48^{c}	14.85 ± 0.75^{a}

Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test. Data represent mean ± standard error (SE) of 10 individual mice; values that do not share a common superscript (a,b,c) differ significantly at $p \leq 0.05$. SND, standard normal chow diet group (Control, low chow diet group); HFD, high fat diet group; HFD + EPF, high fat diet and the extracts from *P. fortunei* flowers (50 or 100 mg/kg).

3.3. Liver Damage Parameters

To determine whether EPF can attenuate the liver damage in the HFD mice, we measured the aminotransferase activities. As shown in Table 3, Fat-rich diet led to higher serum aminotransferase activities of ALT (83.3%), AST (42.3%) as compared with the control group, but these effects were blocked by EPF supplementation. No significant differences in the aminotransferase activities in serum were found between the normal diet supplied with the EPF group and the control group.

Table 3. Effect of EPF on serum biochemical parameters of mice.

Parameter	SND	HFD	HFD + EPF (50 mg/kg)	HFD + EPF (100 mg/kg)	SND + EPF (100 mg/kg)
ALT (U/L)	28.32 ± 1.38^{a}	51.91 ± 2.16^{b}	42.36 ± 1.83^{c}	36.51 ± 2.11^{d}	28.43 ± 2.07^{a}
AST (U/L)	42.86 ± 2.14^{a}	60.97 ± 1.83^{b}	44.73 ± 3.21^{c}	43.68 ± 2.54^{c}	42.89 ± 3.19^{a}
Glucose (mM)	7.65 ± 0.48^{a}	15.38 ± 2.05^{b}	11.12 ± 1.25^{c}	9.93 ± 1.01^{d}	7.68 ± 0.27^{a}
Insulin (mU/L)	3.38 ± 0.17^{a}	5.45 ± 1.32^{b}	4.26 ± 0.42^{c}	3.82 ± 0.24^{d}	3.39 ± 0.23^{a}
HOMA-IR	1.16 ± 0.02^{a}	3.73 ± 0.04^{b}	2.11 ± 0.05^{c}	1.69 ± 0.03^{d}	1.16 ± 0.01^{a}
TC (mM)	2.92 ± 0.21^{a}	7.06 ± 0.34^{b}	5.27 ± 0.19^{c}	4.46 ± 0.32^{d}	2.91 ± 0.16^{a}
TG (mM)	0.83 ± 0.11^{a}	1.57 ± 0.14^{b}	1.19 ± 0.12^{c}	0.97 ± 0.09^{d}	0.82 ± 0.14^{a}
HDL (mM)	1.59 ± 0.15^{a}	1.36 ± 0.13^{b}	1.49 ± 0.11^{c}	1.53 ± 0.12^{a}	1.59 ± 0.21^{a}
LDL (mM)	0.41 ± 0.06^{a}	2.63 ± 0.21^{b}	1.67 ± 0.13^{c}	1.54 ± 0.17^{c}	0.40 ± 0.09^{a}

Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test. Data represent mean ± standard error (SE) of seven individual mice; values that do not share a common superscript (a,b,c,d) differ significantly at $p \leq 0.05$. SND, standard normal chow diet group (Control, low chow diet group); HFD, high fat diet group; HFD + EPF, high fat diet and the extracts from *P. fortunei* flowers (50 or 100 mg/kg).

3.4. Plasma Glucose and Insulin Concentrations

As shown in Table 3, a HFD induced marked elevations of blood glucose (101.1%), plasma insulin (61.2%) and HOMA-IR (169.8%), while EPF treatment markedly lowered fasting plasma insulin, glucose and HOMA-IR relative to HFD mice. No significant differences in blood glucose, plasma insulin and HOMA-IR were found between the normal diet supplied with the EPF group and the control group.

3.5. Serum Lipid Profiles

As expected, mice fed with HFD exhibited a significant hyperlipidemia characterized by increased TC (141.8%), TG (89.2%) and LDL (541.5%) levels in serum compared with these of the control group, respectively (Table 3). Notably, EPF treatment markedly decreased serum TC, TG and LDL levels in

HFD mice after eight weeks ($p < 0.01$). Moreover, HDL level was significantly increased ($p < 0.05$) in the group co-treated with HFD and EPF compared with the animals only treated with HFD ($p < 0.05$). No significant differences in blood lipid levels were found between the normal diet supplied with the EPF group and the control group.

3.6. Hepatic Lipid Accumulation

In HFD group, the levels of liver weight and hepatic TG were increased by 37.4% and 144.8% as compared with those of the control group, respectively ($p < 0.01$). However, EPF treatment reduced hepatic lipid accumulation and liver weight (Figure 1B,C). No significant differences in liver TG content were found between the normal diet supplied with the EPF group and the control group. Tissue sections stained with oil red O represented that a signification increase in the hepatic levels of lipid deposition in HFD-fed mice compared with those of the control group. Likewise, EPF treatment attenuated hepatic lipid accumulation, consistent with liver TG content (Figure 1A).

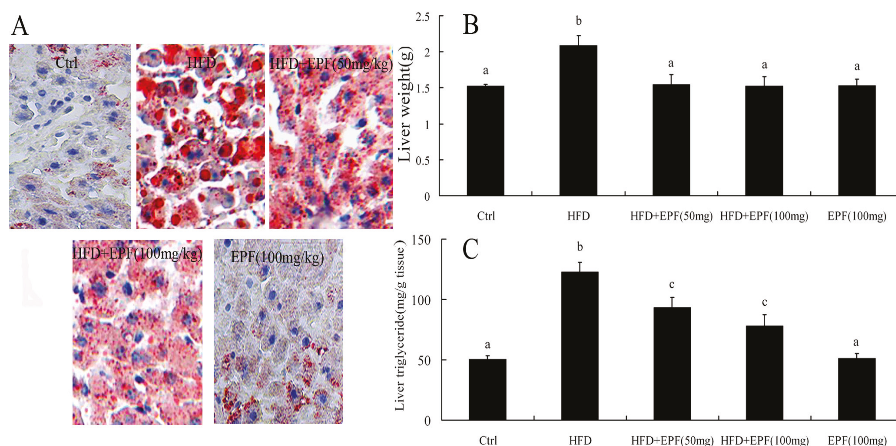


Figure 1. Hepatic lipid accumulation, liver weight and histological evaluation in mice: (**A**) the status of hepatic lipid accumulation in the different rat groups was analyzed after liver section staining with the Oil Red O method; (**B**) liver weight; and (**C**) liver triglyceride. SND, standard normal chow diet group (Control, low chow diet group); HFD, high fat diet group; HFD + EPF, high fat diet and the extracts from *P. fortunei* flowers (50 or 100 mg/kg). Original magnification, ×200. Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test Values are mean ± the standard error of the mean (SEM) ($n = 3$). Values that do not share a common superscript (a,b,c) differ significantly at $p \leq 0.05$.

3.7. Hepatic AMPK Activation

To reveal the potential mechanisms of EPF action, we examined the AMPK phosphorylation levels in livers of HFD-fed mice. Figure 2 showed that the AMPK phosphorylation levels significantly decreased in livers of HFD-fed mice. However, the levels of phosphorylated AMPK were reduced by treatment with EPF (50 or 100 mg/kg).

3.8. AMPK Activation in HepG2 Cells

We further examined the AMPK phosphorylation levels in HepG2 cells. As shown in Figure 3, the AMPK phosphorylation levels continuously increased until 8 h in HepG2 cells, when treated with 100 µg/mL EPF (Figure 3A). Moreover, HepG2 cells were treated with 10–100 µg/mL EPF for 4 h. expression levels of phosphorylated AMPK increased in a dose-dependent manner. However,

after treatment with compound C (an AMPK inhibitor), the phosphorylated AMPK levels in HepG2 cells were significantly reduced (Figure 3B). These results clearly show that EPF treatment leads to AMPK phosphorylation.

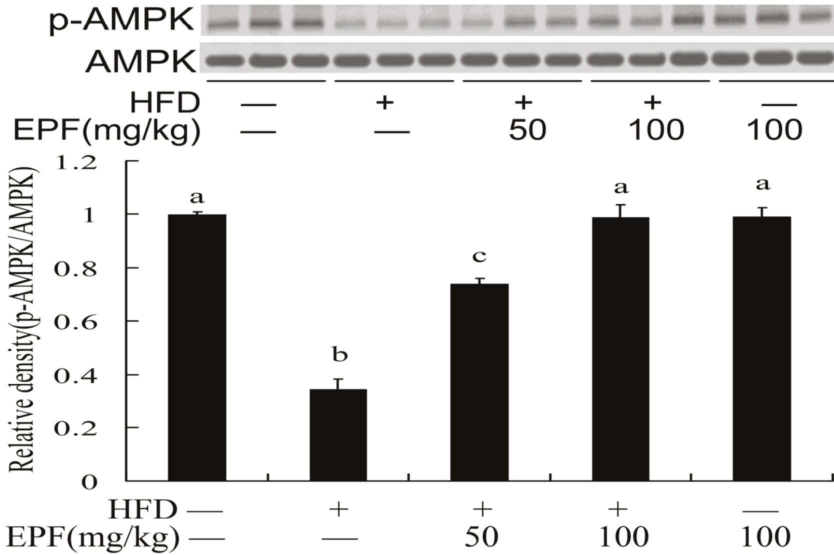


Figure 2. EPF increased AMPK activations in livers of HFD-fed. The vehicle control is set as 1.0. Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test. Each value is expressed as mean ± SEM (n = 3). Values that do not share a common superscript (a,b,c,d) differ significantly at $p \leq 0.05$.

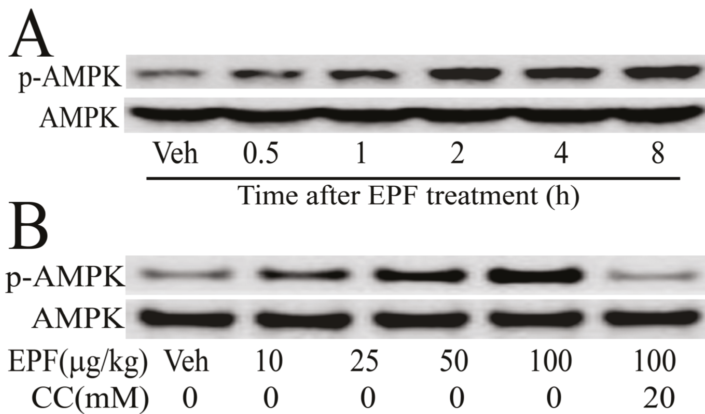


Figure 3. Western blot analysis of protein expression levels of AMPK in HepG2 cells: (**A**) cellular extracts were collected at the indicated times after treatment of EPF (100 μg/mL); and (**B**) cellular extracts were collected at 4 h after EPF treatment (10–100 μg/mL).

3.9. AMPK Inhibition Reduces the Effect of EPF on Lipid Accumulation in FFA-Exposed Hepatocytes

We then examined the effects of AMPK activation on lipid accumulation in FFA-exposed hepatocytes. HepG2 cells were treated with compound C 30 min prior to EPF treatment. Lipid accumulation in HepG2 cells was measured 24 h after FFA exposure using Oil Red O staining. EPF markedly inhibited lipid accumulation in FFA-exposed HepG2 cells at a concentration of 100 μg/mL. Moreover, FFA exposure markedly increased intracellular TG levels in HepG2 cells by 78.8% as compared with these of the control group, but these effects were blocked by compound C (Figure 4).

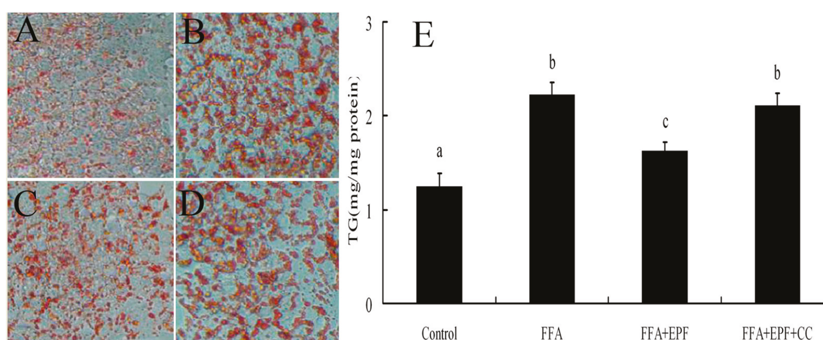


Figure 4. AMPK inhibition reduces the effect of EPF on lipid accumulation in FFA (free fatty acids) exposed hepatocytes: (**A**) control cells; (**B**) FFA treated cells; (**C**) FFA + EPF treated cells; (**D**) FFA + EPF + Compound C treated cells; amd (**E**) the level of intracellular TG in HepG2 cells. The cells were treated with 1 mM FFA mixture for 24 h and EPF (100 μg/mL) was treated 1 h prior to FFA mixture exposure. The cells were stained with Oil Red O and analyzed using a spectrometer at 545 nm. DMSO (0.1%) was treated as a vehicle for EPF, and control cells were treated only with 1% BSA. Compound C (CC) was pretreated 30 min prior to EPF treatment. Photographs (Original magnification, ×400) are representative images of 3 independent experiments. Values that do not share a common superscript (a,b,c) differ significantly at $p \leq 0.05$ (DMRT).

3.10. Hepatic Expressions of Proteins Associated with Lipid Metabolism in Livers

To investigate the mechanisms through which EPF regulated hepatic lipid accumulation, we further evaluated the expressions of proteins associated with lipid metabolism in livers. As shown in Figure 5, the expression levels of sterol regulatory element binding protein 1c (SREBP-1c), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and fatty acid synthase (FAS) were markedly up-regulated in the livers of HFD group as compared with the control group. However, EPF supplementation down-regulated the expression levels of SREBP-1c protein, and its target genes ($p < 0.01$). The carnitine palmitoyltransferase 1 (CPT1) expression level was also decreased in the livers of HFD group as compared with the control group. Interestingly, EPF supplementation significantly enhanced the CPT1 expression in the livers of HFD group ($p < 0.01$).

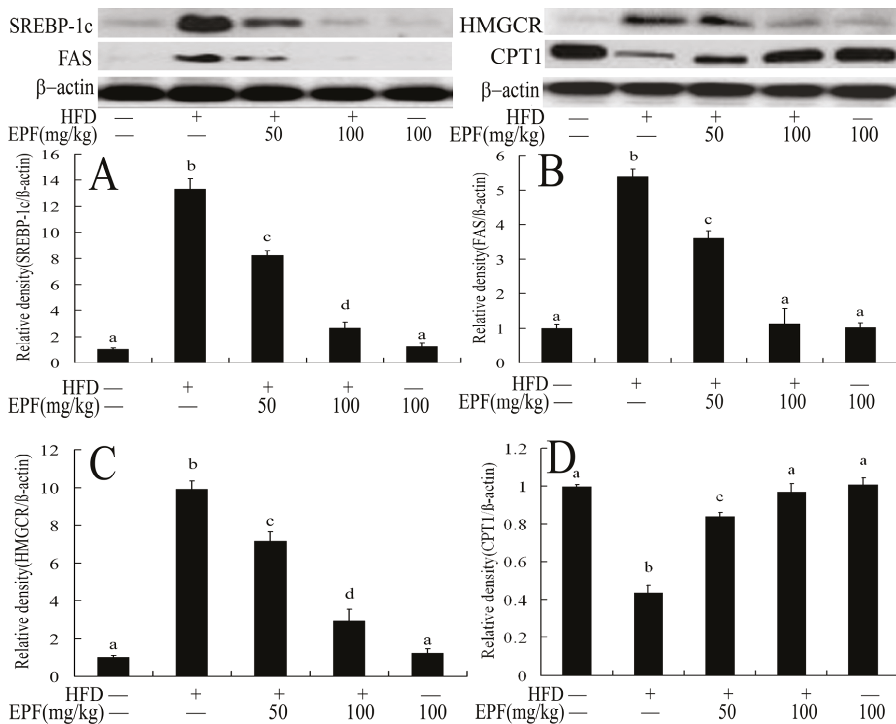


Figure 5. Western blot analysis of protein expression levels in association with the hepatic lipid metabolism in mice: (**A**) relative density analysis of HREBP-1c; (**B**) relative density analysis of FAS; (**C**) relative density analysis of HMGCR; and (**D**) relative density analysis of CPT1. β-Actin was probed as an internal control in relative density analysis. The vehicle control is set as 1.0. Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test. Each value is expressed as mean ± SEM (n = 7). Values that do not share a common superscript (a,b,c,d) differ significantly at $p \leq 0.05$.

3.11. Hepatic Activation of IRS-1

The activation of insulin receptor substrates 1 (IRS1) played important role in blood glucose regulation in livers. To investigate the mechanisms through which EPF inhibited insulin resistance in HFD group, we evaluated the IRS-1(Ser 307) phosphorylation level in mouse livers. As shown in Figure 6, the IRS-1(Ser 307) phosphorylation levels were markedly increased in the livers of HFD group as compared with the control group. Interestingly, EPF supplementation significantly enhanced the IRS-1(Tyr 307) phosphorylation in the livers of HFD-fed mice ($p < 0.01$).

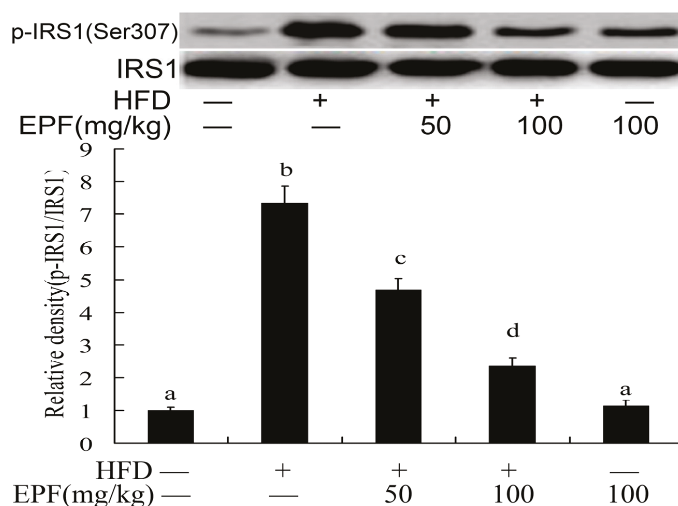


Figure 6. Western blot analysis of IRS1 phosphorylation levels in the livers of mice. The relative density is expressed as the ratio (Phospho-IRS1/IRS1). The vehicle control is set as 1.0. Significant differences among the groups were assessed by one-way ANOVA with Tukey's post hoc test. Each value is expressed as mean ± SEM (n = 7). Values that do not share a common superscript (a,b,c) differ significantly at $p \leq 0.05$.

4. Discussion

The flower of *P. fortunei* has long been used a medicine and food source in China. However, detailed information related to the beneficial effects of its polyphenol composition remains scarce. Here, we aimed to evaluate the protective effects of extract from *P. fortunei* flowers (EPF) on insulin resistance and lipid metabolism disorders in livers. We found that flower extracts of *P. fortunei* (EPF) contain high amounts of flavonoids. The current study clearly indicated that EPF inhibited hyperlipidemia, hepatic lipid accumulation and insulin resistance in obese mice by AMPK pathway.

Several studies had revealed that high fat diet could cause hepatic steatosis, hyperlipidemia, hyperinsulinemia, obesity, insulin resistance and other lipid metabolism disorder disease in humans and in laboratory animals [1,2]. The results of the present study showed that high-fat diet led to significant increase in body weight, hyperlipidemia, hyperglycemia, hyperinsulinemia, and hepatic lipid accumulation, which were in agreement with previous studies [1,2,23]. Phenolic compounds, which are widespread in plants, showed therapeutic effects to obesity and other HFD-induced disease [1,24]. In this study, as shown in Table 2, we observed that supplementation with EPF (50 and 100 mg/kg) significantly deceased body weight by 23.42% and 31.26% compared with HFD mice, respectively, which suggested that EPF has the anti-obesity effects.

High fat diet could induce liver damage [2,25,26]. Previous research found that the flower extracts of the Scrophulariaceae family plants had protective effects against hepatotoxicity [26]. Consistently, we found that EPF supplementation significantly decreased the diagnostic indicators of liver damage as compared with the fat-rich diet (Table 3). These results suggest susceptibility of EPF to the harmful action of the fat-rich diet, and the protective effect of EPF.

It is well known that long-term HFD could induce hyperglycemia and elevations of blood insulin levels. Moreover, increased insulin secretion is in part related to pancreatic islet hyperplasia with progression of insulin-resistance by HFD supply [5,22]. In the present study, we found that blood glucose, insulin levels and HOMA-IR increased significantly ($p \leq 0.05$) in the HFD group compared to the control group, revealing insulin resistance in the HFD group (Table 3). Furthermore, this rising

blood glucose levels may be attributed to the large accumulation of fat in the liver and the enhancement of gluconeogenesis induced by hepatic cells, reducing the transformation of glucose into fat caused by lipodystrophy [27,28]. However, EPF supplementation in HFD-induced obesity mice for eight weeks markedly ameliorated insulin resistance conditions, as indicated by lower serum glucose and insulin levels at the end of the experiment compared to the HFD group, which is thought to stimulate the pancreatic insulin secretion from the β cells of the islets of langerhans and aldose reductase enzyme inhibitory activity [20]. The rich flavonoids (apigenin, luteolin, rutin and hesperetin) in EPF might play important role in the anti-obesity and hypoglycemic effects [24,29,30]. The decreased insulin levels in mice supplied with EPF may be attributed to reduced body weight gain and fat accumulation in livers. Moreover, the protective effects of EPF against obesity and lipid accumulation could also be attributed to the prevention of hyperlipidemia, hyperglycemia in mice fed with HFD. Thus, EPF could inhibit insulin-resistance or conduce to the classical diabetes mellitus treatment.

Previous research from our laboratory and others illustrated that several flavonoids including puerarin, quercetin, hesperetin, epicatechin, apigenin and anthocyanins could reduced serum lipid levels [14,15,24,31]. Previous reports have revealed different results regarding the change in serum TG, TC and LDL-C levels of ICR mice treated with HFD for eight weeks [32–34]. Present observations are in agreement with previous demonstrations that HFD increased the levels of TG, TC and LDL-C [33,34]. Interestingly, EPF treatment markedly decreased serum TC, TG and LDL levels and increased HDL levels in HFD mice after eight weeks, which suggested that flavonoids might be the main bioactive compounds in EPF that exhibited the beneficial effects on the lipid profile (Table 3). Furthermore, the digestion and intestinal absorption of the flavonoids apigenin, luteolin, quercetin and hesperetin from EPF might lower hepatic lipid accumulation suppressing hepatic lipogenesis and lipid absorption [29–31]. We observed that EPF treatment significantly decreased liver weight and liver triglyceride content of mice in a dose-dependent manner. Histological analysis showed that EPF supplementation markedly reduced lipid vacuoles and lipid droplets in livers of HFD-fed mice (Figure 1), consistent with liver TG content.

AMPK plays important roles in regulating energy status and lipid metabolism, which is also potential therapeutic target for many prevalent diseases, including diabetes, obesity, and high blood pressure [3,4]. Many studies showed that several natural compounds, including resveratrol, epigallocatechin gallate, berberine, and quercetin could inhibit lipid-related metabolic disorders by regulating the AMPK activition and its target genes [3,4,19]. In this study, EPF markedly stimulated the AMPK activation in livers of mice and in HepG2 cells (Figures 2 and 3). This result suggested that AMPK pathway may be involved in the modulation of hepatic lipid metabolism in livers of HFD-fed mice and HepG2 cells treated with EPF. Consistent with this regulation, the AMPK inhibitor compound C blocked the effects of EPF on lipid accumulation in HepG2 cells, demonstrating that AMPK activation is necessary for the modulation of hepatic lipid metabolism (Figure 4).

AMPK can mediate lipid metabolism in livers by regulating the expression levels its target genes SREBP-1c, FAS, ACC and HMGCR [3,4,19]. Previous report showed that AMPK coordinates the long-term adaptation of lipid metabolism by regulating the transcriptional factor SREBP-1c (a key transcription factor involved in the control of cholesterol and fatty acid synthesis), which further increase the transcription of FAS and SCD-1, resulting in an increase in the synthesis of TG [3,4,19,35]. Several studies have demonstrated that the flavonoid extracted from plants could inhibit lipid-related metabolic disorders by regulating the expression levels of AMPK and SREBP-1c and their target genes [1,22,29,36]. As demonstrated in our Western blot analysis, EPF supplementation significantly down-regulated the expression levels of AMPK and SREBP-1c and their target gene HMGCR, an endoplasmic reticulum bound and peroxisomal enzyme that is the rate-limiting step in cholesterol biosynthesis, suggesting that EPF had a positive effect due to the lower expression of HMGCR and contributed to inhibit the serum cholesterol increase due to high fat diet (Figure 5). Moreover, EPF treatment significantly down-regulated the expression level of FAS, a key enzymes involved in lipogenesis and up-regulated the expression level of carnitine palmitoil transferase 1 (CPT1), which

catalyzes the entrance of fatty acids into the mitochondria and it is the rate limiting enzyme of hepatic fatty acid β-oxidation (Figure 5). Therefore, these results implied that the reduced biosynthesis and enhanced fatty acid oxidation might contribute to the beneficial effects of EPF on hyperlipidemia and hepatic lipid accumulation in the HFD mice. These data explained the anti-obesity, hypolipidemic and hypoglycemic effects of EPF to a certain extent.

As previously expressed, insulin resistance is another key pathophysiological feature and pathogenesis in high fat diet mice [2,20,21]. The hyperlipidemia and hyperglycemia induced by high fat diet could decrease the number of insulin receptors, glucose transport and metabolism thereby increasing insulin resistance and reducing insulin sensitivity [23]. Insulin signaling is a cascade of events initiated by the activation of insulin receptor substrates (IRS). AMPK could regulate glucose metabolism insulin resistance by IRS pathway [37]. Insulin resistance could also decrease the AMPK phosphorylation and increase the expression levels of SREBP-1c, which further suppressed IRS-1 activation, inhibits IRS-1-associated insulin signaling and thereby decreases glucose uptake and utilization [3,4,19,38]. To understand the molecular mechanisms contributing to insulin resistance, we further observed the effects of EPF supplementation on these key factors of insulin signaling in liver tissue (Figure 6). Consistent with these reports, our results showed HFD feeding for 8 weeks increased the SREBP-1c expression levels and inhibited its downstream molecule IRS-1 (Ser 307) phosphorylation, which substantiates the development of insulin resistance in the livers of mice. However, EPF dietary supplementation partially or completely prevented all these alterations associated with HFD consumption, suggesting that EPF might be a natural flavonoid-rich extract and the activator of IRS-1, which in turn regulates the expression of downstream genes involved in hepatic glucose and lipid metabolism to lower blood glucose and lipid.

5. Conclusions

This is the first report that the extracts from *P. fortunei* flowers have potent protective effects against hyperlipidemia, hepatic lipid accumulation and insulin resistance in HFD mice and the protective effects of EPF, at least in part, were associated with the decreased lipogenesis, increased glucose metabolism and induced fatty acid oxidation in livers by AMPK pathway. We propose a possible protective effect of EPF (Figure 7). Our results emphasize the importance of dietary intervention in the treatment and/or prevention of diseases induced by HFD. Although, seven flavonoid compounds were identified in the extract from *P. fortunei* flowers by HPLC analysis, we were not clear if all of them were bioactive agents. Therefore, this question warrants further investigation.

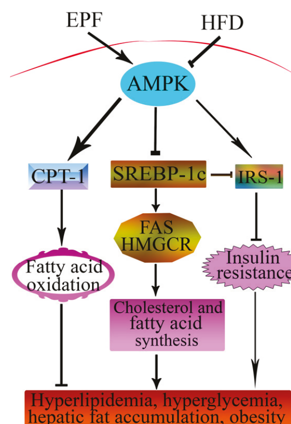


Figure 7. Schematic diagram showing protective signaling of EPF in livers of HFD-fed mice. The → indicates activation or induction, and ⊣ indicates inhibition or blockade

Acknowledgments: This work is supported from the Natural Science Project Foundation of Jiangsu Province (BK20141147), grants from the Fund of Sichuan Province Key Laboratory of Brewing Biotechnology and Application (NJ2014-17) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Author Contributions: Chanmin liu and Jieqiong Ma conceived and designed the experiments; Jianmei Sun and Wei Yang performed the experiments; Zhaojun Feng and Chao Cheng analyzed the data; Hong Jiang contributed reagents/materials/analysis tools; Chanmin Liu wrote the paper.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
AMPK	AMP-activated kinase
CPT1	the carnitine palmitoyltransferase 1
EPF	the extract from *Paulownia fortunei* flowers
FAS	fatty acid synthase
HFD	high fat diet
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
IRS-1	insulin receptor substrate 1
SREBP-1c	sterol regulatory element binding protein 1c

References

1. Wang, S.; Moustaid-Moussa, N.; Chen, L.; Mo, H.; Shastri, A.; Su, R.; Bapat, P.; Kwun, I.; Shen, C.L. Novel insights of dietary polyphenols and obesity. *J. Nutr. Biochem.* **2014**, *25*, 1–18. [CrossRef] [PubMed]
2. Yki-Järvinen, H. Nutritional modulation of non-alcoholic fatty liver disease and insulin resistance. *Nutrients* **2015**, *7*, 9127–9138. [CrossRef] [PubMed]
3. Hardie, D.G.; Ross, F.A.; Hawley, S.A. AMP-activated protein kinase: A target for drugs both ancient and modern. *Chem. Biol.* **2012**, *19*, 1222–1236. [CrossRef] [PubMed]
4. Hwang, J.T.; Kwon, D.Y.; Yoon, S.H. AMP-activated protein kinase: A potential target for the diseases prevention by natural occurring polyphenols. *New Biotechnol.* **2009**, *26*, 17–22. [CrossRef] [PubMed]
5. Firenzuoli, F.; Gori, L. Herbal medicine today: Clinical and research issues. *Evid. Based Complement. Altern. Med.* **2007**, *4*, 37–40. [CrossRef] [PubMed]
6. Šmejkal, K.; Svačinová, J.; Šlapetová, T.; Schneiderová, K.; Dall'Acqua, S.; Innocenti, G.; Závalová, V.; Kollár, P.; Chudík, S.; Marek, R.; et al. Cytotoxic activities of several geranyl-substituted flavanones. *J. Nat. Prod.* **2010**, *73*, 568–572. [CrossRef] [PubMed]
7. Gao, T.Y.; Jin, X.; Tang, W.Z.; Wang, X.J.; Zhao, Y.X. New geranylated flavanones from the fruits of *Paulownia catalpifolia* Gong Tong with their anti-proliferative activity on lung cancer cells A549. *Bioorg. Med. Chem. Lett.* **2015**, *25*, 3686–3689. [CrossRef] [PubMed]
8. Jin, Q.; Lee, C.; Lee, J.W.; Lee, D.; Kim, Y.; Hong, J.T.; Kim, J.S.; Kim, J.H.; Lee, M.K.; Hwang, B.Y. Geranylated Flavanones from *Paulownia coreana* and their inhibitory effects on nitric oxide production. *Chem. Pharm. Bull.* **2015**, *63*, 384–387. [CrossRef] [PubMed]
9. Cho, J.K.; Curtis-Long, M.J.; Lee, K.H.; Kim, D.W.; Ryu, H.W.; Yuk, H.J.; Park, K.H. Geranylated flavonoids displaying SARS-CoV papain-like protease inhibition from the fruits of *Paulownia tomentosa*. *Bioorg. Med. Chem.* **2013**, *21*, 3051. [CrossRef] [PubMed]
10. Navrátilová, A.; Nešuta, O.; Vančatová, I.; Čížek, A.; Varela-M, R.E.; López-Abán, J.; Villa-Pulgarin, J.A.; Mollinedo, F.; Muro, A.; Žemličková, H.; et al. C-Geranylated flavonoids from *Paulownia tomentosa* fruits with antimicrobial potential and synergistic activity with antibiotics. *Pharm. Biol.* **2016**, *54*, 1398–1407. [CrossRef] [PubMed]
11. Cho, J.K.; Ryu, Y.B.; Curtis-Long, M.J.; Ryu, H.W.; Yuk, H.J.; Kim, D.W.; Kim, H.J.; Lee, W.S.; Park, K.H. Cholinestrase inhibitory effects of geranylated flavonoids from *Paulownia tomentosa* fruits. *Bioorg. Med. Chem.* **2012**, *20*, 2595. [CrossRef] [PubMed]

12. Zhang, P.F.; Li, C. Flavones from flowers of *Paulownia fortunei*. *Zhongguo Zhong Yao Za Zhi* **2008**, *33*, 2629–2632. [PubMed]
13. Li, X.Q.; Zhang, P.F.; Duan, W.D.; Zhang, D.L.; Li, C. Studies on the chemical constituents from Flower of *Paulownia fortunei*. *Zhong Yao Cai* **2009**, *32*, 1227–1229. [PubMed]
14. Liu, C.M.; Ma, J.Q.; Sun, Y.Z. Protective role of puerarin on lead-induced alterations of the hepatic glutathione antioxidant system and hyperlipidemia in rats. *Food Chem. Toxicol.* **2011**, *49*, 3119–3127. [CrossRef] [PubMed]
15. Ma, J.Q.; Ding, J.; Zhao, H.; Liu, C.M. Puerarin attenuates carbon tetrachloride-induced liver oxidative stress and hyperlipidaemia in mouse by JNK/c-Jun/CYP7A1 pathway. *Basic Clin. Pharmacol. Toxicol.* **2014**, *115*, 389–395. [CrossRef] [PubMed]
16. Samad, N.B.; Debnath, T.; Jin, H.L.; Lee, B.R.; Park, P.J.; Lee, S.Y.; Lim, B.O. Antioxidant activity of *Benincasa hispida* seeds. *J. Food Biochem.* **2013**, *37*, 388–395. [CrossRef]
17. Sakakibara, H.; Honda, Y.; Nakagawa, S.; Ashida, H.; Kanazawa, K. Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *J. Agric. Food Chem.* **2003**, *51*, 571–581. [CrossRef] [PubMed]
18. De Las Heras, N.; Valero-Muñoz, M.; Martín-Fernández, B.; Ballesteros, S.; López-Farré, A.; Ruiz-Roso, B.; Lahera, V. Molecular factors involved in the hypolipidemic- and insulin-sensitizing effects of a ginger (*Zingiber officinale* Roscoe) extract in rats fed a high-fat diet. *Appl. Physiol. Nutr. Metab.* **2017**, *42*, 209–215. [CrossRef] [PubMed]
19. Seo, M.S.; Hong, S.W.; Yeon, S.H.; Kim, Y.M.; Um, K.A.; Kim, J.H.; Kim, H.J.; Chang, K.C.; Park, S.W. Magnolia of officinalis attenuates free fatty acid-induced lipogenesis via AMPK phosphorylation in hepatocytes. *J. Ethnopharmacol.* **2014**, *157*, 140–148. [CrossRef] [PubMed]
20. Porto, L.C.; da Silva, J.; Ferraz, A.B.; Ethur, E.M.; Porto, C.D.; Marroni, N.P.; Picada, J.N. The antidiabetic and antihypercholesterolemic effects of an aqueous extract from pecan shells in wistar rats. *Plant Foods Hum. Nutr.* **2015**, *70*, 414–419. [CrossRef] [PubMed]
21. Naowaboot, J.; Wannasiri, S.; Pannangpetch, P. Morin attenuates hepatic insulin resistance in high-fat-diet-induced obese mice. *J. Physiol. Biochem.* **2016**, *72*, 269–280. [CrossRef] [PubMed]
22. Yan, F.; Dai, G.; Zheng, X. Mulberry anthocyanin extract ameliorates insulin resistance by regulating PI3K/AKT pathway in HepG2 cells and db/db mice. *J. Nutr. Biochem.* **2016**, *36*, 68–80. [CrossRef] [PubMed]
23. Sudhakara, G.; Mallaiah, P.; Sreenivasulu, N.; Sasi Bhusana Rao, B.; Rajendran, R.; Saralakumari, D. Beneficial effects of hydro-alcoholic extract of *Caralluma fimbriata* against high-fat diet-induced insulin resistance and oxidative stress in Wistar male rats. *J. Physiol. Biochem.* **2014**, *70*, 311–320. [CrossRef] [PubMed]
24. Ragab, S.M.M.; Elghaffar, S.K.A.; El-Metwally, T.H.; Badr, G.; Mahmoud, M.H. Effect of a high fat, high sucrose diet on the promotion of non-alcoholic fatty liver disease in male rats: The ameliorative role of three natural compounds. *Lipids Health Dis.* **2015**, *14*, 83. [CrossRef] [PubMed]
25. Zhang, F.; Hartnett, S.; Sample, A.; Schnack, S.; Li, Y. High fat diet induced alterations of atrial electrical activities in mice. *Am. J. Cardiovasc. Dis.* **2016**, *6*, 1–9. [PubMed]
26. Kochi, T.; Shimizu, M.; Terakura, D.; Baba, A.; Ohno, T.; Kubota, M.; Shirakami, Y.; Tsurumi, H.; Tanaka, T.; Moriwaki, H. Non-alcoholic steatohepatitis and preneoplastic lesions develop in the liver of obese and hypertensive rats: Suppressing effects of EGCG on the development of liver lesions. *Cancer Lett.* **2014**, *342*, 60–69. [CrossRef] [PubMed]
27. El-Domiaty, M.M.; Wink, M.; Abdel Aal, M.M.; Abou-Hashem, M.M.; Abd-Alla, R.H. Antihepatotoxic activity and chemical constituents of *Buddleja asiatica* Lour. *Z. Naturforsch. C* **2009**, *64*, 11–19. [CrossRef] [PubMed]
28. Si, X.; Zhou, Z.; Strappe, P.; Blanchard, C. A comparison of RS4-type resistant starch to RS2-type resistant starch in suppressing oxidative stress in high-fat-diet-induced obese rats. *Food Funct.* **2017**, *8*, 232–240. [CrossRef] [PubMed]
29. Kwon, E.Y.; Jung, U.J.; Park, T.; Yun, J.W.; Choi, M.S. Luteolin attenuates hepatic steatosis and insulin resistance through the interplay between the liver and adipose tissue in mice with diet-induced obesity. *Diabetes* **2015**, *64*, 1658–1669. [CrossRef] [PubMed]
30. Jung, U.J.; Cho, Y.Y.; Choi, M.S. Apigenin ameliorates dyslipidemia, hepatic steatosis and insulin resistance by modulating metabolic and transcriptional profiles in the liver of high-fat diet-induced obese mice. *Nutrients* **2016**, *8*, 305. [CrossRef] [PubMed]

31. Hoek-van den Hil, E.F.; van Schothorst, E.M.; van der Stelt, I.; Swarts, H.J.; van Vliet, M.; Amolo, T.; Vervoort, J.J.; Venema, D.; Hollman, P.C.; Rietjens, I.M.; et al. Direct comparison of metabolic health effects of the flavonoids quercetin, hesperetin, epicatechin, apigenin and anthocyanins in high-fat-diet-fed mice. *Genes Nutr.* **2015**, *10*, 469. [CrossRef] [PubMed]
32. Li, Z.; Jin, H.; Oh, S.Y.; Ji, G.E. Anti-obese effects of two Lactobacilli and two Bifidobacteria on ICR mice fed on a high fat diet. *Biochem. Biophys. Res. Commun.* **2016**, *480*, 222–227. [CrossRef] [PubMed]
33. Zhu, X.; Zhang, W.; Zhao, J.; Wang, J.; Qu, W. Hypolipidaemic and hepatoprotective effects of ethanolic and aqueous extracts from *Asparagus officinalis* L. by-products in mice fed a high-fat diet. *J. Sci. Food Agric.* **2010**, *90*, 1129–1135. [CrossRef] [PubMed]
34. Jeong, E.J.; Jegal, J.; Ahn, J.; Kim, J.; Yang, M.H. Anti-obesity effect of *Dioscorea oppositifolia* extract in high-fat diet induced obese mice and its chemical characterization. *Biol. Pharm. Bull.* **2016**, *39*, 409–414. [CrossRef] [PubMed]
35. Horton, J.D.; Goldstein, J.L.; Brown, M.S. SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Investig.* **2002**, *109*, 1125–1131. [CrossRef] [PubMed]
36. Liu, G.; Zhang, Y.; Liu, C.; Xu, D.; Zhang, R.; Cheng, Y.; Pan, Y.; Huang, C.; Chen, Y. Luteolin alleviates alcoholic liver disease induced by chronic and binge ethanol feeding in mice. *J. Nutr.* **2014**, *144*, 1009–1015. [CrossRef] [PubMed]
37. Jayanthy, G.; Roshana Devi, V.; Ilango, K.; Subramanian, S.P. Rosmarinic acid mediates mitochondrial biogenesis in insulin resistant skeletal muscle through activation of AMPK. *J. Cell. Biochem.* **2017**, *118*, 1839–1848. [CrossRef] [PubMed]
38. Bi, Y.; Wu, W.; Shi, J.; Liang, H.; Yin, W.; Chen, Y.; Tang, S.; Cao, S.; Cai, M.; Shen, S.; et al. Role for sterol regulatory element binding protein-1c activation in mediating skeletal muscle insulin resistance via repression of rat insulin receptor substrate-1 transcription. *Diabetologia* **2014**, *57*, 592–602. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Could Pomegranate Juice Help in the Control of Inflammatory Diseases?

Francesca Danesi [1,*] and Lynnette R. Ferguson [2]

[1] Department of Agri-Food Science and Technology (DISTAL), University of Bologna, Piazza Goidanich 60, 47521 Cesena, Italy
[2] Discipline of Nutrition and Dietetics, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand; l.ferguson@auckland.ac.nz
* Correspondence: francesca.danesi@unibo.it; Tel.: +39-0547-338957

Received: 30 July 2017; Accepted: 26 August 2017; Published: 30 August 2017

Abstract: Fruits rich in polyphenols, such as pomegranates, have been shown to have health benefits relating to their antioxidant and anti-inflammatory properties. Using data obtained from PubMed and Scopus, this article provides a brief overview of the therapeutic effects of pomegranate on chronic inflammatory diseases (CID) such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), metabolic and cardiovascular disorders, and other inflammatory-associated conditions, with an emphasis on fruit-derived juices. Most studies regarding the effects of pomegranate juice have focused on its ability to treat prostate cancer, diabetes, and atherosclerosis. However, pomegranate juice has shown therapeutic potential for many other illnesses. For instance, a small number of human clinical trials have highlighted the positive effects of pomegranate juice and extract consumption on cardiovascular health. The beneficial effects of pomegranate components have also been observed in animal models for respiratory diseases, RA, neurodegenerative disease, and hyperlipidaemia. Furthermore, there exists strong evidence from rodent models suggesting that pomegranate juice can be used to effectively treat IBD, and as an anti-inflammatory agent to treat CID. The effects of pomegranate intake should be further investigated by conducting larger and more well-defined human trials.

Keywords: pomegranate; *Punica granatum*; pomegranate juice; ellagitannins; inflammation; inflammatory diseases; anti-inflammatory properties

1. Introduction

Inflammation is a complex biological response to tissue injury and infection. Chronic inflammation has been shown to be involved in the onset and development of a range of disorders. Chronic inflammatory disease (CID) is a general term used for conditions where persistent inflammation plays a central role in disease pathology [1]. Examples of CID include rheumatoid arthritis (RA), inflammatory bowel disease (IBD), chronic obstructive pulmonary disease (COPD), asthma, and psoriasis. Patients with CID present with heavy infiltration of inflammatory cells at the site of disease—e.g., joints, intestinal mucosa, lungs, and skin—and show elevated levels of inflammatory mediators [2]. Dysfunctional inflammatory responses have also been implicated as contributors to other chronic diseases such as atherosclerosis, type 2 diabetes, obesity, insulin resistance, and certain neurodegenerative diseases (e.g., Alzheimer's disease) [3].

Ellagitannins (ET) and ellagic acid (EA) are polyphenols present in some fruits, nuts, and seeds—such as pomegranates, black raspberries, raspberries, strawberries, walnuts, and almonds [4]. It has been shown that ET-rich fruits have anti-oxidant, anti-inflammatory, anti-neoplastic, and chemo-preventive properties [5]. Pomegranate (*Punica granatum* L.) is a rich source of ET punicalagin

(Figure 1), which aroused considerable interest in pomegranate fruit as a novel therapeutic within the last several years (Figure 2).

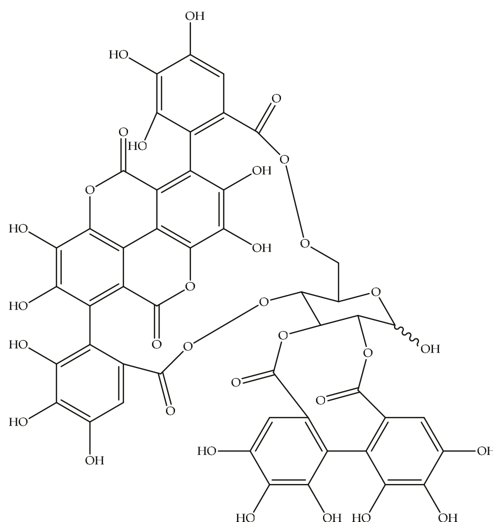


Figure 1. The structure of punicalagin, the main polyphenolic compound present in pomegranate.

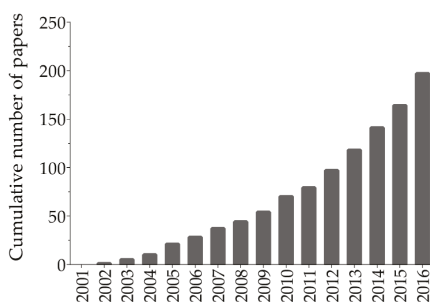


Figure 2. The popularity of health benefits of ellagitannins over time as shown by the cumulative number of papers from 2001 to 2016 obtained by a basic search on the PubMed database using "ellagitannins" and "health" as terms search.

Although it is widely accepted that pomegranate intake can provide significant health benefits, the results of human clinical trials using pomegranate juice as a therapeutic agent have been inconsistent. This may, in part, be due to variability in the composition of the administered pomegranate products. Several studies have suggested that pomegranate intake has positive effects on blood pressure [6,7] and cardiovascular risk in diabetic [8,9], obese [10,11], hypertensive and ischemic patients [12,13]. Conversely, a meta-analysis found that pomegranate had no effect on lipid profiles [14]. In addition, a meta-analysis of data from five prospective trials did not find a significant effect of pomegranate juice on plasma C-reactive protein (CRP) levels [15].

No clear consensus has yet emerged on the putative anti-inflammatory effects of pomegranate intake on CID. Therefore, we conducted a systematic review to provide an overview of the evidence of the potential benefits of pomegranate products—with an emphasis on fruit-derived juices—on this occurrence.

2. Search Strategy

We performed an extensive search using the PubMed and Scopus databases in April 2017. The following keywords and Medical Subject Headings (MeSH) terms were combined: "pomegranate" or "Punica granatum", "inflam*", and "disease*". We did not use language restrictions, and reviews were excluded. The search strategies were as follows: Medline search strategy (pomegranate*) OR (Punica granatum) AND inflam* AND disease* NOT review*; Scopus search strategy (TITLE-ABS-KEY (pomegranate*) OR TITLE-ABS-KEY (Punica granatum) AND TITLE-ABS-KEY (inflam*) AND TITLE-ABS-KEY (disease*)) AND (LIMIT-TO (DOCTYPE, "ar")).

3. Results and Discussion

The literature selection process was conducted following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) recommendations [16]. The initial search yielded 156 hits after the exclusion of duplicates. During the screening process (reviewing of titles and abstracts), 25 records were excluded. After full-text analysis, another 76 papers were excluded. Altogether, 55 papers were selected for detailed evaluation. Both reviewers independently selected the evaluated articles. Figure 3 shows the flow chart of the selection procedure of the papers.

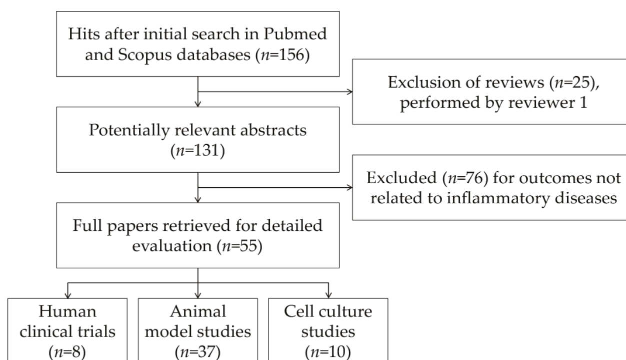


Figure 3. Flow chart of papers included in the review.

Human clinical trials are relatively few (Table 1) and were mainly focused on the effects of pomegranate consumption on cardiovascular health.

Table 1. Published research studies on the potential beneficial effects on Chronic Inflammatory Disease (CID) of pomegranate products.

CID	Human Clinical Trials (No. of Subjects)	Animal Model Studies	Cell Culture Studies
Asthma and COPD	-	3 [17–19]	-
IBD	-	8 [20–27]	2 [28,29]
Immune system	-	-	2 [30,31]
Metabolic and cardiovascular disorders §	7 (51 [32], 13 [33], 31 [34], 30 [35], 101 [36], 24 [37], 27 [38])	14 [39–52]	-
Neurodegenerative diseases	-	8 [53–60]	4 [53,61–63]
Psoriasis	-	-	-
RA	1 (55 [64])	1 [65]	1 [66]
Other disorders §§	-	3 [67–69]	1 [70]

COPD: chronic obstructive pulmonary disease; IBD: inflammatory bowel disease; RA: rheumatoid arthritis. § atherosclerosis, type 2 diabetes, obesity, metabolic syndrome, insulin resistance, hyperlipidaemia. §§ cell proliferation, hyperplasia, metaplasia, cancer.

3.1. Findings Related to Pomegranate Products Consumption and CID in Humans

Table 2 summarises the data published on human trials related to pomegranate intake. Unfortunately, there exists a limited number of human trials available concerning the effects of pomegranate on the outcomes of patients with CID. Within these studies, we found the tested dietary products and experimental designs to be highly variable. Only one trial has been conducted using pomegranate seed oil [32], whereas most other studies have been carried out using either pomegranate juice [33–37] or the commercially available whole fruit phenolic extract POMx^{TM} [37,38,64], which are both safe and well-tolerated by CID patients. The doses of pomegranate juice or extract used also varied from study to study as groups used a range of 100 to 500 mL [35] of juice and 0.5 to 1 g of POMx^{TM} [37,38]. Additionally, the study designs used differ for each trial—some used pre- and post-test schemes [33,34] or randomised placebo-controlled tests [32,35,36,38,64], and one trial even lacked an appropriate placebo comparator [37]. Finally, the duration of each study is not consistent—some performed acute (1 day) [33] or short-term (1 week) [35] trials and others assessed the long-term (6–12 months) effects of pomegranate intake [36,38]. While these studies focused primarily on patients affected by metabolic and cardiovascular disorders [32–38] and RA [64], there exist three clinical trials that are currently in progress exploring the therapeutic potential of pomegranate juice on IBD, memory impairment, ageing and skin inflammation (Table 3).

Even though the use of differing approaches renders it difficult to draw general conclusions, there is a generally positive effect of pomegranate consumption in patients with chronic inflammatory disorders. Pomegranate juice appears to have promising hypotensive properties in patients with hypertension [33] or metabolic syndrome [35,71], and in patients undergoing dialysis [38]. It also resulted in a slight amelioration of lipid profiles in patients with cardiovascular disease (CVD), as pomegranate intake elevated endogenous levels of high-density lipoprotein (HDL)-cholesterol [34] and reduced triglyceride (TG) levels [32]. However, several studies have been unable to confirm pomegranate's TG- and cholesterol-lowering effect [34,35,37,38]. With regards to risk factors for CVD, the consumption of 150 mL of pomegranate juice—restricted to one serving per day—did not influence the level of circulating soluble adhesion molecules or markers of atherosclerosis and subclinical coronary heart disease (CHD) in hypertensive individuals [33]. Evidence for the beneficial effects of pomegranate on CVD was obtained using long-term consumption (100 mL daily for one year) [36] or with a greater intake of juice (500 mL daily) [35].

Markers of systemic inflammation have not been consistently evaluated in many of these studies, a factor which has an impact on the conclusions drawn from each trial. It has been shown that circulating pro-inflammatory cytokines in patients affected by cardiovascular disorders are slightly reduced by the consumption of pomegranate juice and extract. Notably, a decrease in the level of interleukin 6 (IL-6)—a well-known pro-inflammatory cytokine—has been demonstrated in patients with type 2 diabetes [34] and in patients undergoing haemodialysis [36]. Conversely, pomegranate intake has no effect on the plasma levels of CRP or tumour necrosis factor α (TNF-α), except in patients with metabolic syndrome [35] or those undergoing dialysis [36]. It is generally accepted that the anti-inflammatory effects of pomegranate intake are mediated by its anti-oxidant properties. In agreement, pomegranate intake results in an improvement in plasma antioxidant capacity, as it has been shown to decrease the prevalence of oxidatively damaged molecules and increase anti-oxidant-dependent immune responses in patients affected by CID [34,64].

In conclusion, evidence from human trials indicates that pomegranate, when administered as a juice in high doses or for an extended period, can reduce oxidative stress and systemic inflammation. However, there exist limitations to many of these studies as these trials were limited in sample size, used short durations of supplementation, and/or lacked necessary controls.

Table 2. Summary of findings related to pomegranate products consumption and CID in humans.

Study Design	Population	Subjects (Gender, No., Age)	Intervention	Control/Comparator	Duration	Outcomes	Reference
Double-blind, placebo-controlled, randomised	Dyslipidaemic patients	F & M: 51, 42–64 years	Pomegranate seed oil 400 mg/day × 2	Paraffin 400 mg/day × 2	4 weeks	↓ TG, ↓ HDL-C, ↓ TG/HDL-C ratio, ↔ TNF-α	Asghari et al., 2012 [32]
Pre- and post-test	Hypertensive patients	M: 13, 39–68 years	Pomegranate juice 150 mL/day	-	1 day	↓ SBP, ↓ DBP, ↔ CRP, ↔ ICAM-1, ↔ VCAM-1, ↔ IL-6, ↔ E-selectin	Asgary et al., 2013 [33]
Pre- and post-test	Type 2 diabetic patients	F: 16, M: 15, 38–54 years	Concentrated pomegranate juice 50 g/day	-	4 weeks	↔ SBP, ↔ DBP, ↑ TC, ↑ HDL-C, ↔ TG, ↔ LDL-C, ↔ glycaemia, ↓ IL-6, ↔ TNF-α, ↔ CRP, ↓ adiponectin, ↑ TAC	Shishehbor et al., 2016 [34]
Double-blind, placebo-controlled, randomised crossover	Patients with metabolic syndrome	F: 16, M: 14, 42–62 years	Pomegranate juice 500 mL/day	Placebo 500 mL/day	1 week	↓ SBP, ↓ DBP, ↓ CRP, ↑ TG, ↑ VLDL-C	Moazzen & Alizadeh 2017 [35]
Double-blind, placebo-controlled, randomised	Haemodialysis patients	F: 46, M: 55, 55–81 years	Pomegranate juice 100 mL/day	Placebo 100 mL/day × 1	12 months	↓ IL-6, ↓ TNF-α, ↓ MPO, ↓ AOPP, ↓ oxidised fibrinogen, ↓ MDA	Shema-Didi et al., 2012 [36]
Pilot, open, randomised crossover	Haemodialysis patients	F: 13, M: 11, 47–75 years	Pomegranate juice 100 mL/day; Pomegranate extract POMx™ 1050 mg/day (both containing 650 mg GAE)	-	4 weeks	↔ SBP, ↔ DBP, ↔ CRP, ↔ IL-6, ↔ F2-isoprostanes, ↔ isofurans, TG, ↔ TC, ↔ HDL-C, ↔ LDL-C	Rivara et al., 2015 [37]
Double-blind, placebo-controlled, randomised, parallel-arm	Haemodialysis patients	F: 10, M: 17, 49–59 years	Pomegranate extract POMx™ 1 g/day (containing 600–755 mg GAE)	Placebo 1 pill/day	6 months	↓ SBP, ↓ DBP, ↔ CRP, ↔ IL-6, ↔ TC, ↔ HDL-C, ↔ LDL-C, ↔ TG, ↔ ORAC, ↔ AOPP, ↔ 8-OHdG, ↔ ox-LDL, ↔ arylesterase activity, ↔ lactonase activity, ↔ PON activity	Wu et al., 2015 [38]

Table 2. *Cont.*

Study Design	Population	Subjects (Gender, No., Age)	Intervention	Control/Comparator	Duration	Outcomes	Reference
Double-blind, placebo-controlled, randomised	RA patients	F & M: 55, 37–61 years	Pomegranate extract POMx^{TM} 250 mg/day × 2	Placebo (cellulose) 250 mg/day × 2	8 weeks	↔ CRP, ↔ MMP3, ↔ MDA, ↑ GPx, ↓ ESR, ↓ DAS28, ↓ HAQ, ↓ swollen joints, ↓ tender joints, ↓ pain intensity, ↓ morning stiffness	Ghavipour et al., 2017 [64]

↑: increase; ↔ : no change; ↓: decrease; 8-OHdG: 8-hydroxy-20-deoxyguanosine; AOPP: advanced oxidation protein products; CRP: C-reactive protein; DAS28: disease activity score; DBP: diastolic blood pressure; ESR: erythrocyte sedimentation rate; F: female; GAE: gallic acid equivalents; GPx: glutathione peroxidase; HAQ: health assessment questionnaire; HDL-C: high-density lipoprotein–cholesterol; ICAM-1: intracellular adhesion molecule-1; IL-6: interleukin 6; LDL-C: low-density lipoprotein–cholesterol; M: male; MDA: malondialdehyde; MMP3: matrix metalloproteinase-3; MPO: myeloperoxidase; ORAC: oxygen radical absorbance capacity; ox-LDL: oxidised low-density lipoprotein; PON: paraoxonases; SBP: systolic blood pressure; TAC: total antioxidant capacity; TC: total cholesterol; TG: triglycerides; TNF-α: tumour necrosis factor α; VCAM-1: vascular cell adhesion molecule-1; VLDL-C: very low-density lipoprotein–cholesterol.

Table 3. Ongoing clinical trials on pomegranate juice [72].

clintrials.gov Identifier	Study Focus	Study Design, Duration	Sponsor	Estimated Enrolment	Study Start Date	Estimated Completion Date
NCT02093130	Memory in older adults	Double-blind, placebo-controlled, parallel arm, randomised, 12 months	University of California (Los Angeles, CA, USA)	212	January 2014	December 2017
NCT02258776	Ageing and inflammation of the skin	Single-blind, placebo-controlled, parallel arm, randomised, 12 weeks	University of California (Los Angeles, CA, USA)	15	October 2015	January 2018
NCT03000101	Inflammation in IBD	Double-blind, placebo-controlled, parallel arm, randomised, 12 weeks	St. Orsola-Malpighi Hospital (Bologna, Italy)	36	December 2016	June 2018

3.2. Evidence of Anti-Inflammatory Effects of Pomegranate or Pomegranate-Derived Products in Different Animal Models of CID

Animal models have been used to investigate the pathology of a wide range of chronic inflammatory diseases—COPD, IBD, metabolic and cardiovascular disorders, neurodegenerative diseases, RA, cancer—in which inflammation is induced experimentally by genetic manipulation or pharmacologically (diet, drugs, or exogenous toxicants) (Table 4). These studies examined the effects of different pomegranate-related products, including whole fruit juice [19,21,26,41–43,48,50,60] or extract [22,24,26,40,43,44,52,55,56,59,67–69], extracts obtained using various parts of the fruit (e.g., peel [17,45,47,58], seeds [20,43,46,50,54], flowers [27,45], leaves [18]), concentrated extracts (POMx™ [65], Pomanox® [51]) and bioactive molecules found in pomegranates (punicalagin [53], EA [23–25,49,57], punicic acid [20]) and their derived metabolites (urolithins [22]). The administered doses of these extracts varied in range between 10 to 80 mg/kg [24,25,57]. Based on the previous findings of Kaulmann and Bohn (2016) [73], the doses administered in these in vivo studies probably resulted in supra-physiological serum concentrations of pomegranate metabolites obtained only through supplements in humans.

These studies found anti-inflammatory effects of pomegranate and its biologically active compounds. The inflammation-related endpoints measured varied between local—e.g., macroscopic and histological examinations—to systemic evaluations—scoring systems, and plasma cytokines and CRP levels. Inflammation can also be induced by oxidative stress and, accordingly, oxidative markers were often evaluated. Among them, the most commonly measured endpoints included the formation of malondialdehyde (MDA) [22,26,27,53,54,58,68], antioxidant capacity (measured as FRAP or ferric reducing ability of plasma) [22], and the activities of glutathione peroxidase (GPx) [48], superoxide dismutase (SOD) [26,42,48,52,58,68], catalase (CAT) [48,68], and myeloperoxidase (MPO) [20,24–27]. Typically, the reduction of local inflammation and oxidative stress has also been reported to have more systemic effects.

Administration of pomegranate-derived products has been shown to reduce local inflammation in the bronchoalveolar tissue of COPD model mice [17–19] and in the joints of RA model mice [65]. There also exists a strong base of evidence suggesting that pomegranate extract exerts anti-inflammatory effects that may alleviate the symptoms of IBD [20–27], as colon tissue damage [20,22–27], antioxidant status [20,22,24–27], and inflammation [21–26] were all ameliorated by pomegranate fruit supplementation in rodent models of IBD. The mechanisms involved appear to be related to the inhibition of NF-κB [23–26], c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and signal transducer and activator of transcription 3 (STAT3) phosphorylation [23–25] in colon tissue.

Several groups have studied the effects of pomegranate on the prevention and amelioration of atherosclerosis and other CVD symptoms. de Nigris et al. (2007) [43] reported that supplementation with pomegranate juice or pomegranate fruit extract decreased the expression of vascular inflammation markers and transforming growth factor β-1 (TGFβ-1), and, likewise, increased endothelial NO synthase (eNOS) levels in a rat model of metabolic syndrome. Additionally, Labsi et al. (2016) [74] showed that intraperitoneal treatment with pomegranate peel extract for two months after the induction of echinococcosis significantly reduced the nitric oxide (NO) and TNF-α levels in Swiss albino mice. Pomegranate juice supplementation also ameliorated cardiac hypertrophy, and reduced oxidative stress and expression levels of interleukin 1β (IL-1β) and several fibrotic markers in the aorta of rats exposed to cigarette smoke [39]. Similarly, pomegranate juice supplementation slowed the development of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) in a diet-induced obesity rat model [48]. Other authors illustrated the hypoglycaemic activity of pomegranate flowers [45], seeds [46], peel [45], and juice [41,48] in addition to its bioactive compound EA [49] using various rodent models of diabetes. Here, pomegranate-derived products appeared to regulate the activation of peroxisome proliferator-activated receptor γ (PPAR-γ) [46], a known regulator of fatty acid storage and glucose metabolism.

Table 4. Overview of the anti-inflammatory effects of pomegranate or pomegranate-derived products in animal models of CID.

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
Respiratory diseases	BalbC mice	Pomegranate peel aqueous extract (200 mg/kg b.w.) via intraperitoneal injection for 2 days	LPS-induced lung inflammation	↓ total cells in BAL, ↓ neutrophils in BAL	Bachoual et al., 2011 [17]
	BalbC mice	Encapsulated pomegranate leave extract (10 mg/mL) or non-encapsulated pomegranate leave extract (20 mg/kg b.w.) via nostril for 4 days	Ovalbumin-induced asthma	↓ leukocytes, neutrophils, and eosinophils in BAL, ↓ macrophages in BAL (non-encapsulated extract only), ↔ lymphocytes in BAL, ↓ IL-1β and IL-5 in BAL	de Oliveira et al., 2013 [18]
	C57BL/6J mice	Pomegranate juice (80 µmol/kg b.w.) via bottle for 1 week or 1 month or 3 months	Cigarette smoke-induced lung stress	↓ IL-1β and IL-6 expression in lung (1 week only), ↓ TNF-α expression in lung	Husari et al., 2016 [19]
IBD	Wistar rats	Punicic acid (400 mg/0.5 mL PBS) or pomegranate seed oil (0.5 mL) via oral administration for 10 days	TNBS-induced colitis	Punicic acid: ↓ Wallace and Ameho scores, ↓ MPO activity in colon, ↓ F2-isoprostane in colon; Pomegranate seed oil: ↓ Wallace and Ameho scores	Boussetta et al., 2009 [20]
	Swiss albino mice	Pomegranate flower hydro-alcoholic extract (100 or 200 mg/kg b.w.) or EA-rich fraction of pomegranate flower (100 or 200 mg/kg b.w.) via oral administration for 7 days	DSS-induced colitis	↓ macroscopic and histopathological changes in colon, ↓ colon MPO activity, ↓ histamine content in colon, ↓ MDA level in colon, ↓ superoxide anion production in colon	Singh et al., 2009 [27]
	Fischer rats	Pomegranate extract (250 mg/kg b.w.) or urolithin A (15 mg/kg b.w.) via chow for 10 days	DSS-induced colitis	↓ colon tissue damage (urolithin A only), ↑ FRAP (pomegranate extract only), ↓ MDA in colon (pomegranate extract only), ↓ COX-2 gene and protein expression in colon, ↓ iNOS expression in colon, ↓ PGE_2 and NO levels in colon (pomegranate extract only), ↓ PTGES protein expression in colon	Larrosa et al., 2010 [22]
	Wistar rats	EA (10 and 20 mg/kg b.w.) via oral gavage for 48, 24 and 1 h prior to the induction of colitis and 24 h later	TNBS-induced colitis	↓ colon macroscopic damage, ↓ b.w. loss, ↓ colon weight/length, ↓ histological damage in colon, ↓ colon MPO activity, ↓ iNOS and COX-2 protein expression in colon, ↓ JNK and ERK phosphorylation in colon, ↓ NF-κB activation in colon	Rosillo et al., 2011 [25]

Table 4. *Cont.*

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
	Wistar rats	Pomegranate extract (250 or 500 mg/kg feed) or EA (10 mg/kg feed) or EA-enriched pomegranate extract (pomegranate extract 250 mg/kg feed + EA 10 mg/kg feed) via chow for 30 days prior to the induction of colitis and 14 days later	TNBS-induced colitis	↓ colon macroscopic damage, ↓ b.w. loss (all treatments, apart from extract 250 mg/kg), ↓ colon weight/length (EA and EA-enriched extract only), ↓ colon MPO activity, ↓ TNF-α level in colon, ↓ iNOS and COX-2 protein expression in colon, ↓ JNK and ERK phosphorylation in colon, ↓ NF-κB activation in colon, ↔ colon PPAR-γ protein expression	Rosillo et al., 2012 [24]
	C57BL/6 mice	EA (0.5% *w*/*w*, equivalent to 25 mg/mouse) via chow for 56 days	DSS-induced colitis	↓ disease symptoms, ↓ DAI, ↓ iNOS and COX-2 protein expression in colon, ↓ JNK and ERK phosphorylation in colon, ↓ NF-κB activation in colon, ↓ IL-6 gene expression in colon, ↓ STAT3 phosphorylation in colon	Marín et al., 2013 [23]
	Sprague-Dawley rats	Pomegranate beverage (containing 2504.74 mg/L GAE) *ad libitum* for 3 weeks prior to the induction of colitis and 7 weeks later	DSS-induced colitis	↓ colonocyte proliferative index, ↓ expression of hs-CRP, TNF-α, IL-1β, and IL-6 in intestinal mucosa, ↓ IL-1β and IL-6 levels in serum, ↑ IL-10 level in serum, ↓ p-p70-S6K/p70-S6K, ↓ p-rpS6/rpS6	Kim et al., 2016 [21]
	Sprague-Dawley rats	Pomegranate juice (400 mg/kg b.w.) or pomegranate powder (4 mg/kg b.w.) via oral administration for 18 days	DNBS-induced colitis	↔ histopathological scores, ↓ CMDI and DAI, ↓ MDA in colon (juice only), ↔ colon MPO activity, ↓ colon NO production, ↓ colon SOD activity, ↓ serum cortisol level, ↓ IL-1β, IL-18, TNF-α, and NF-κB expression in colon	Shah et al., 2016 [26]
Metabolic and cardiovascular disorders	Zucker rats	Concentrated pomegranate juice or pomegranate fruit extract (6.25 mL/L) via drinking water or pomegranate seed oil (1 mL/L) via chow for 5 weeks	Obese metabolic syndrome model	↔ TC, ↔ LDL-C, ↔ HDL-C, ↑ TG (seed oil only), ↔ daytime MAP, ↔ BPM, ↔ motor activity, ↓ arterial TSP-1 protein expression, ↑ eNOS protein expression (apart from oil), ↓ arterial TGF-β1 protein expression (except oil), ↓ nitrate and nitrite levels (apart from oil), ↔ insulin and glucose levels	de Nigris et al., 2007 [43]
	db/db mice	Pomegranate seed oil (1 g/100 g feed) via chow for 30 days	Diabetes and obesity model	↓ glycaemia, ↓ blood insulin, ↑ expression of genes PPAR-α, CD36, and FABP4 in adipose tissue, ↔ expression of genes PPAR-γ, ACAD, and SCD1 in adipose tissue, ↑ expression of genes PPAR-γ, CD36, FABP4, ACAD, and SCD1 in muscle, ↔ expression of genes PPAR-α in muscle, ↓ TNF-α expression and NF-κB activation in adipose tissue and liver	Hontecillas et al., 2009 [46]

Table 4. *Cont.*

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
	CD-1 mice	Pomegranate juice (12.5 mL/L juice diluted in water, equivalent to 0.35 mmol polyphenols) via drinking water for 4 months	Streptozotocin-induced diabetes	↑ hepatic PON-1 expression and activity, ↓ glycaemia, ↔ blood TC and TG levels	Betanzos-Cabrera et al., 2011 [41]
	Sprague-Dawley rats	Pomegranate juice (100 µL) via gastric gavage for 10 weeks	Streptozotocin-induced diabetes	↔ GSH in lung, ↑ SOD activity in lung, ↓ protein carbonyl content in lung, ↓ serum sialic acid, ↓ eNOS protein in lung	Çukurova et al., 2012 [42]
	SR-BI/apoE double knockout mice	Pomegranate extract (307.5 ml/L) via drinking water for 2 weeks	Coronary heart disease model	↑ TC, ↔ serum apoA and apoB, ↓ atherosclerosis, ↔ SAA and serum MCP-1, ↓ MCP-1 in plaques, ↓ lipid accumulation, macrophage infiltration, and MCP-1 levels in heart, ↓ myocardial fibrosis, cardiac enlargement, and ECG abnormalities	Al-Jarallah et al., 2013 [40]
	BalbC mice	Pomegranate peel extract (0.2% *w*/*v* diluted in water, equivalent to 6 mg per mouse) via drinking water for 4 weeks	High-fat diet-induced obesity and hypercholesterolaemia	↔ body weight gain, ↔ adiposity, ↔ glycaemia and insulin response, ↓ serum TC and LDL-C, ↔ serum HDL-C and TG, ↔ hepatic TC and TG, ↔ IL-1β, IL-6, and COX-2 expression in liver, ↔ IL-1β expression in colon, ↓ IL-6 and COX-2 expression in colon	Neyrinck et al., 2013 [47]
	Wistar rats	EA (0.8 g/kg feed) via chow for 8 weeks after the induction of metabolic syndrome	High-fat and high-carbohydrate diet-induced metabolic syndrome	↑ retroperitoneal, epididymal, omental, and total abdominal fat, ↔ whole-body fat mass, ↓ whole-body lean mass, ↓ glycaemia, ↓ plasma TG, TC, NEFA, uric acid, urea, and CRP, ↓ plasma ALT, AST, ALP, and LDH activity, ↔ plasma albumin and bilirubin, ↓ SBD, ↑ coronary endothelial-dependent relaxation, ↔ Nrf2 protein expression in heart, ↑ Nrf2 protein expression in liver, ↓ NF-κB expression in heart and liver, ↑ CPT1 expression in heart and liver	Panchal et al., 2013 [49]
	Wistar Albino Glaxo rats	Pomegranate extract (300 mg/kg b.w.) via chow for 8 weeks	High-fat diet-induced metabolic syndrome	↔ weight of epididymal adipose tissue, ↔ glycaemia, ↓ LDL-C, ↔ TC, HDL-C, TG, and FFA, ↔ SBP, ↓ serum corticosterone, ↔ adrenal corticosterone, ↓ serum IL-6 and TNF-α, ↓ TG in liver	Dushkin et al., 2014 [44]

Table 4. *Cont.*

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
	Sprague-Dawley rats	PUNI-enriched pomegranate extract (150 mg/kg b.w.) via oral gavage for 8 weeks	High-fat diet-induced NAFLD	↓ body weight gain, ↓ serum TG, HDL-C, and LDL-C, ↔ serum C, ↓ serum insulin, leptin, and adiponectin, ↓ HOMA-IR, ↓ serum ALT level, ↓ liver tissue weight, ↓ hepatic TG and TC, ↓ expression of SREBP-1c precursor protein, ↔ expression of SREBP-1c mature protein, expression of FA biosynthesis-related genes (↓ SREBP-1c, ↓ FAS, ↓ ACC1, ↓ SCD1), expression of TG biosynthesis-related genes (↓ ACLY, ↔ GPAM, ↑ DGAT-1 and-2), ↓ serum CRP level, IL-1β, IL-4, IL-6, and TNFα, ↓ serum IgA, IgG, and IgM, ↓ protein carbonyl content in liver tissue and liver mitochondria, ↓ lipid peroxidation in liver, ↑ hepatic total SOD activity, ↓ hepatic GSH and GSSG levels, ↑ GSH/GSSG ratio, ↓ Nrf2, HO-1, NQO-1, and UCP2 protein expression in liver, ↑ ATP content in liver, ↑ activities of mitochondrial complexes I, II, and IV in liver, ↑ expression of genes PGC-1-α and PPAR-α in liver, ↔ expression of PGC-1β gene in liver, ↑ PGC-1α protein expression in liver, ↑ expression of genes CPT1A, CPT1B, and ACAD in liver	Zou et al., 2014 [52]
	Pigs	Pomegranate extract Pomanox® (625 mg equivalent to 200 mg punicalagins) via chow for 10 days	High-fat diet-induced coronary endothelial dysfunction	↑ coronary endothelial-dependent relaxation, ↑ Akt and eNOS phosphorylation in coronary artery, ↔ MCP-1 gene expression in coronary artery, ↓ MCP-1 protein content in coronary artery, ↓ coronary DNA oxidative damage, ↓ LDL-C oxidation	Vilahur et al., 2015 [51]
	Sprague-Dawley rats	Pomegranate juice concentrate (equivalent to 80 µmol polyphenols/mL) via drinking water for 5 weeks	Cigarette smoking-induced cardiac hypertrophy	↔ DBP and SBP, ↓ ROS in aortic tissue, ↓ heart to body weight ratio, ↓ fibrotic marker (ObR and Fn1) and kinin receptor (Bdkrb1 and Bdkrb2) expression in aorta, ↓ IL-1β expression in aorta, ↔ TNF-α expression in aorta	Al Hariri et al., 2016 [39]

Table 4. *Cont.*

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
	C57Bl/6 mice	Pomegranate peel (250 mg/kg b.w.) or Pomegranate flower extract (250 mg/kg b.w.) or Pomegranate seed oil (2 mL/kg b.w.) for 6 weeks	High-fat and high-sugar diet-induced obesity	↔ b.w. gain, ↓ glycaemia (28 days- seed oil treatment only), ↔ plasma insulin level, ↔ plasma TC, HDL-C, and TG, ↔ hepatic ALT and AST, ↔ hepatic TG, ↑ plasma IL-2 (peel extract only), ↓ plasma IL-6 (apart from flower extract), ↑ plasma IL-10 (flower extract only), ↓ plasma TNF-α (apart from peel extract), ↑ IFN-γ (seed oil only)	Harzallah et al., 2016 [45]
	Sprague-Dawley rats	Pomegranate juice (60 mL) via drinking water for 7 weeks	High-fat and high-sugar diet-induced NAFLD	↓ plasma ALT and AST, ↔ plasma GGT and ALP, ↓ glycaemia and insulin, ↓ plasma TG, ↔ plasma TC, HDL-C, and LDL-C, ↓ hepatic IL-1β, IL-6, TNF-α, and TGF-β1 expression, ↑ hepatic IL-10 expression, ↔ GSH level, TBARS level, GR activity, CAT activity, SOD activity in liver, ↑ hepatic GPx activity, ↓ hepatic steatosis and ballooning, ↓ lobular and portal inflammation in liver	Noori et al., 2017 [48]
	Sprague-Dawley rats	Pomegranate juice (1 mL) or pomegranate seed extract (100 mg/mL) via oral administration, by force-feeding for 21 days	Streptozotocin-nicotinamide induced type 2 diabetes	↔ b.w. gain, ↔ glycaemia and plasma insulin level, ↓ TC and TG (juice only), ↔ LDL-C and HDL-C (juice only), ↑ TC, LDL-C, and HDL-C (seed extract only), ↔ TG (seed extract only), ↓ plasma IL-6 and NF-κB levels, ↓ plasma TNF-α level (juice only), ↑ number and size of Islets of Langerhans (juice only)	Taheri Rouhi et al., 2017 [50]
Neurodegenerative diseases	APPswe/PS1dE9 mice	Pomegranate extract (6.25 mL/L) via drinking water for 3 months	Transgenic model overexpressing APP, developing amyloid plaques and progressive cognitive deficits	↑ behavioural performance, ↓ TNF-α in spleen and brain, ↓ NFATc1 activation in spleen and brain, ↑ p-NFATc2/NFATc2 ratio in brain, ↓ p-IκB/IκB ratio in brain, ↓ plaques in brain	Rojanathammanee et al., 2013 [59]
	Lewis rats	Pomegranate juice (juice diluted 1:40 in water, equivalent to ~0.6–0.7 mg polyphenols) via drinking water for 2 weeks	Rotenone-induced degeneration of neurones	↓ rearing behaviour, ↔ postural instability, ↔ catecholamine levels, ↓ dopamine fibres in striatum, ↓ nigral dopaminergic neurones, ↑ nitrotyrosine in substantia nigra, ↑ iNOS induction, ↑ NF-κB activation, ↑ caspase activation, ↔ IL-1β, TNF-α, and COX-2 protein expression	Tapias et al., 2014 [60]
	C57BL/6 mice	Pomegranate seed oil as emulsified nanodroplets (10 µL) via gavage for 10 days	MOG-induced experimental autoimmune encephalomyelitis	↓ demyelination and oxidation of brain lipids, ↓ MDA in brain	Binyamin et al., 2015 [54]

Table 4. *Cont.*

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
	APPsw/Tg2576 mice	Pomegranate fruit (4% *w*/*w*) via chow for 15 months	Transgenic model overexpressing APP, developing amyloid plaques and progressive cognitive deficits	↓ IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, and eotaxin levels in serum, ↓ Aβ-1 40 and 42 levels in brain, ↑ ATP levels the cortex and hippocampus, ↓ IL-1β, IL-6, TNF-α levels in cortex and hippocampus	Essa et al., 2015 [56]
	APPsw/Tg2576 mice	Pomegranate fruit (4% *w*/*w*) via chow for 15 months	Transgenic model overexpressing APP, developing amyloid plaques and progressive cognitive deficits	↓ expression of genes IL-1β, IL-10, TNF-α, IGF-1, iNOS, and CCL2, ↑ BDNF gene expression, ↑ PSD-95, Munc18-1, SNAP25, and synaptophysin protein expression, ↑ p-CaMKIIα/CaMKIIα protein expression, ↑ p-CREB/CREB protein expression, ↑ BECN1 protein expression, ↑ LC3-I and LC3-II protein expression, ↑ Akt and mTOR protein expression, ↑ p70-S6K protein expression, ↔ APP and CTF-α protein expression, ↓ BACE-1, CTF-β, and sAPP-β protein expression, ↔ ADAM-10 and ADAM-17 protein expression	Braidy et al., 2016 [55]
	Wistar rats and mice	EA (10, 30, and 100 mg/kg b.w.) via intraperitoneal injection in a single administration	Scopolamine- and diazepam-induced cognitive impairments	↓ amnesia in EPM and PA tests in mice ([EA] $\geq$ 30 mg/kg), ↓ amnesia in EPM test in rats ([EA] $\geq$ 30 mg/kg)	Mansouri et al., 2016 [57]
	C57Bl/6 mice	Pomegranate peel extract as microparticles (800 mg/kg b.w.) via oral administration for 35 days	Amyloid-β peptide-induced neurodegeneration	↔ locomotor activity in an activity cage, ↔/↑ spatial memory in the Barnes maze, ↓ senile plaques, ↑ BDNF level in cortex and hippocampus, ↓ acetylcholinesterase activity in cortex and hippocampus, ↓ MDA in liver, ↔ SOD activity in hippocampus, cortex and serum, ↓ TNF-α in cortex, ↔ TNF-α in serum	Morzelle et al., 2016 [58]
	ICR mice	PUNI (1.5 mg/kg b.w.) via drinking water for 4 weeks	LPS-induced cognitive impairment	↓ Aβ and BACE-1 protein expression, ↓ GFAP and AIF-1 protein expression, ↓ IL-1β, IL-6, and TNF-α release, ↑ GSH/GSSG ratio, ↓ ROS level, ↓ MDA, ↓ IκB phosphorylation, ↓ p50 and p65 protein expression	Kim et al., 2017 [53]
RA	DBA/1 Lac J mice	POMx^{TM} extract (13.6 or 34 mg/kg b.w.) via oral gavage for 10 days	Collagen-induced arthritis with chicken CII (Chondrex)	↓ incidence and delay of arthritis, ↓ synovitis, ↓ pannus formation, ↓ joint degradation, ↓ IL-1β expression in ankle joints (13.6 mg/kg only), IL-6 expression in ankle joints, ↓ TNF-α expression in ankle joints (34 mg/kg only)	Shukla et al., 2008 [65]

Table 4. *Cont.*

Disease Model	Animal Model	Tested Product(s), Vehicle, Duration	Disease Induction	Effects	Reference
Hepatocellular carcinoma	Sprague-Dawley rats	Pomegranate emulsion (1 or 10 g/kg b.w.) via oral gavage for 4 weeks prior to the DENA exposure and 18 weeks later	DENA-induced hepatocarcinogenesis	↓ cyclin D1 expression (10 g/kg only), ↑ Bax/Bcl-2 ratio (10 g/kg only), ↓ β-catenin expression (10 g/kg only), ↑ GSK-3 expression (10 g/kg only)	Bhatia et al., 2013 [67]
Prostatic hyperplasia	Sprague-Dawley rats	Pomegranate fruit extract (25, 50, and 100 mg/kg b.w.) via oral gavage for 4 weeks	Testosterone-induced prostatic hyperplasia	↓ prostate weight, ↓ PAP activity, ↑ GSH, ↔ total glutathione, ↑ SOD activity (100 mg/kg only), ↔ CAT activity, ↓ MDA, ↓ iNOS and COX-2 expression, ↔ AR, NF-κB, ER-α, and p-Akt expression	Ammar et al., 2015 [68]
Prostate cancer	Athymic nude mice	Pomegranate fruit extract (0.1% and 0.2% *w*/*v*) via oral administration for 28–51 days (until the implanted tumour reached to a volume of 1200 mm^3)	Implantation with androgen-responsive CWR22Rn1 cells	↓ PSA secretion	Malik & Mukhtar 2006 [69]

↑: increase; ↔ : no change; ↓: decrease; ACAD: acyl coenzyme A dehydrogenase; ACC1: acetyl-CoA carboxylase 1; ACLY: ATP citrate lyase; ADAM: ADAM metallopeptidase; AIF-1: allograft inflammatory factor 1; Akt: protein kinase B; ALP: alkaline phosphatase; ALT: alanine transaminase; apoA: apolipoprotein A; apoB: apolipoprotein B; apoE: apolipoprotein E; APP: amyloid precursor protein; AR: androgen receptor; AST: aspartate aminotransferase; ATP: adenosine triphosphate; Aβ: amyloid β-peptides; b.w.: body weight; BACE-1: β-secretase 1; BAL: bronchoalveolar lavage; Bax: bcl-2-like protein 4; Bcl-2: B-cell lymphoma 2; Bdkrb: bradykinin receptor; BDNF: brain-derived neurotrophic factor; BECN1: beclin-1; BPM: beats per minute; CaMKIIα: calcium/calmodulin-dependent protein kinase type II α chain; CAT: catalase; CCL2: chemokine (C-C motif) ligand 2; CD36: cluster of differentiation 36; CMDI: colon mucosal damage index; COX-2: cyclooxygenase-2; CPT1: carnitine palmitoyl-transferase 1; CREB: cAMP response element-binding protein; CRP: C-reactive protein; CTF: C-terminal fragment of APP; CWR22Rn1: human prostate cancer cell line; DAI: disease activity index; DBP: diastolic blood pressure; DENA: diethyl-nitrosamine; DGAT: diglyceride acyltransferase; DNBS: 2,4-dinitro benzene sulfonic acid; DSS: dextran sulphate sodium; EA: ellagic acid; ECG: electrocardiogram; eNOS: endothelial nitric oxide synthase; EPM: elevated plus maze; ERK: extracellular signal-regulated kinase; ER-α: oestrogen receptor α; FA: fatty acids; FABP4: fatty acid binding protein 4; FAS: fatty acid synthase; FFA: free fatty acids; Fn1: fibronectin; FRAP: ferric reducing ability of plasma; GAE: gallic acid equivalents; GFAP: glial fibrillary acidic protein; GGT: γ-glutamyl-transferase; GPAM: glycerol-3-phosphate acyltransferase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidised glutathione; h: hour(s); HDL-C: high-density lipoprotein-cholesterol; HO-1: heme oxygenase 1; HOMA-IR: homeostatic model assessment–insulin resistance; hs-CRP: high-sensitivity C-reactive protein; ICR: imprinting control region; IgA: immunoglobulin A; IGF-1: insulin-like growth factor 1; IgG: immunoglobulin G; IgM: immunoglobulin M; IL-1β: interleukin 1β; IL-2: interleukin 2; IL-3: interleukin 3; IL-4: interleukin 4; IL-5: interleukin 5; IL-6: interleukin 6; IL-9: interleukin 9; IL-10: interleukin 10; IL-18: interleukin 18; iNOS: inducible nitric oxide synthase; IκB: inhibitor of NF-κB; JNK: c-Jun N-terminal kinase; LC3: microtubule-associated protein 1α/1β-light chain 3; LC3-I: cytosolic form of LC3; LC3-II: LC3-phosphatidylethanolamine conjugated; LDH: lactate dehydrogenase; LDL-C: low-density lipoprotein-cholesterol; LPS: lipopolysaccharide; m: month(s); MAP: mean arterial pressure; MCP-1: monocyte chemoattractant protein-1; MDA: malondialdehyde; MOG: myelin oligodendrocyte glycoprotein; MPO: myeloperoxidase; mTOR: mechanistic target of rapamycin; Munc-18: syntaxin binding protein 1; NAFLD: non-alcoholic fatty liver disease; NEFA: non-esterified fatty acids; NFATc: nuclear factor of activated T-cells, cytoplasmic; NF-κB: nuclear factor κ light-chain-enhancer of activated B cells; NO: nitric oxide; NQO-1: NAD(P)H quinone dehydrogenase 1; Nrf2: nuclear factor (erythroid-derived-2)-like 2; ObR: leptin receptor; p-: phosphorylated; p50: p50 protein; p65: p 65 protein; p70-S6K: ribosomal protein S6 kinase β-1; PA: passive avoidance; PAP: prostatic acid phosphatase; PBS: phosphate-buffered saline; PGC-1-: peroxisomal proliferator-activated receptor-γ coactivator-1; PGE_2: prostaglandin E2; PON-1: paraoxonase 1; PPAR-α: peroxisome proliferator-activated receptor α; PPAR-γ: peroxisome proliferator-activated receptor γ; PSA: prostate-specific antigen; PSD-95: postsynaptic density protein 95; PTGES: prostaglandin E synthase; PUNI: punicalagin; ROS: reactive oxygen species; rpS6: ribosomal protein S6; SAA: serum amyloid A; sAPP-β: soluble APP-β; SBP: systolic blood pressure; SCD1: stearoyl-coenzyme A desaturase 1; SNAP25: synaptosomal-associated protein 25; SOD: superoxide dismutase; SR-BI: scavenger receptor class B type I; SREBP-1c: sterol regulatory element-binding protein 1c; STAT3: signal transducer and activator of transcription 3; TC: total cholesterol; TG: triglycerides; TGF-β1: transforming growth factor β1; TNBS: 2,4,6-trinitrobenzenesulfonic acid; TNF-α: tumour necrosis factor α; TSP-1: thrombospondin 1; UCP2: mitochondrial uncoupling protein 2; *w*/*v*: weight/volume; *w*/*w*: weight/weight.

Several studies assessed the efficacy of pomegranate fruit as an antiproliferative agent in animal models of prostate hyperplasia and carcinoma. Malik and Mukhtar (2005) [69] demonstrated that, in vivo, oral administration of pomegranate fruit extract results in tumour growth inhibition accompanied by a reduction in serum prostate-specific antigen (PSA) levels. The decreasing of serum PSA levels have also been confirmed in two studies in humans [75,76]. In addition, dietary supplementation of 4% pomegranate extract with a standard chow diet inhibited neuro-inflammation in a transgenic mouse model of Alzheimer's disease (AD). A delay in the formation of senile plaques and the loss of synaptic proteins was also observed [55,56]. Conversely, treatment with pomegranate juice did not protect neuronal degeneration in a separate study that used a rat model of AD, but instead exacerbated neuronal cell death and inflammation [60].

In conclusion, these studies using animal models of inflammation-related disease offer a crucial step in answering phenomenological questions related to the pathology of CID, but cannot be fully applied clinically due to the relatively high doses of pomegranate products used.

3.3. In Vitro Anti-Inflammatory Activity of Pomegranate Extracts or Pomegranate-Derived Bioactive Compounds

The anti-inflammatory effects observed in vivo have generally been confirmed in vitro. However, experimental conditions applied to cell cultures also tend to vary considerably with regards to the concentration of pomegranate extracts and time-points used (Table 5). In vitro, several pomegranate products have been tested including whole juice [61,63,70], extracts from the husk [29], seeds [62], or pulp [61,77], bioactive compounds present in pomegranate juice (ET [29,53,77] and EA [30]), or POMx^{TM} extract [31]. Of all the groups working with these compounds, only Giménez-Bastida et al. (2012) [28] decided to assess the effects in intestinal cells of EA, which is present at high concentrations in the human colon. It is well known that pomegranate ETs are first hydrolysed to EA followed by transformation into the metabolite—urolithin—in the gut [5]. The importance of identifying bioactive metabolites using cell-based experimentation has been recently elaborated upon by Aragonès et al. (2017) [78].

Throughout our literature review, we found that the concentrations of the compounds applied to cells (mainly ET and EA) ranged from 1 to 100 μM; concentrations like 100 μM are considered high but physiologically attainable in the gut. The duration of supplementation was also variable, as groups used short-term (1–2 h) and long-term exposure to ET, EA, and pomegranate extracts (24–48 h), and were tested prior to exposure or in combination with pro-inflammatory stimuli, such as IL-1β [28,29,63], TNF-α [28,66], interferon γ (IFN-γ) [29], lipopolysaccharide (LPS) [29,53,62], phorbol 12-myristate 13-acetate (PMA) [31,79], or glucose deprivation [61]. In a few cases, pomegranate extracts or bioactive molecules were tested individually [30,70].

As mentioned above, in vivo studies have defined a clear role for NF-κB in the modulation of inflammation by pomegranate extracts, a finding that appears to be confirmed in vitro. Pomegranate juice [63], POMx^{TM} extract [31], and their bioactive compounds—punicalagin [53] or delphinidin [66]—all suppressed NF-κB activation in various types of cells. It was found that ET reduced the expression of NF-κB target genes, including IL-6 and interleukin 8 (IL-8), upon exposure to pro-inflammatory stimuli in intestinal cells [29], while EA [30] and POMx^{TM} [31] reduced NF-κB activation in various subsets of immune cells, and anthocyanin delphinidin reduced inflammation in rheumatoid arthritis cells [66]. Taken together, these results suggest that ET and other bioactive compounds present in pomegranate juice show anti-inflammatory effects in vitro, and that the mechanisms involved appear to be related to inactivation of NF-κB signalling.

Table 5. Summary of the anti-inflammatory effects of pomegranate extracts or pomegranate-derived bioactive compounds assayed in cell culture studies.

Cell Model	Primary Cell/Cell Line	Tested Compound(s), Dose, Duration	Pro-Inflammatory Treatment	Biological Effects	Reference
Intestinal cells	CCD18-Co	Uro-A (40 μM) + Uro-B (5 μM) + EA (1 μM) for 12–48 h in concomitant exposure with pro-inflammatory stimulus	IL-1β (1 ng/mL) or TNF-α (50 ng/mL)	↓ IL-8 release, ↓ PGE_2 release (only upon IL-1β stimulus), ↓ PAI-1 release, ↔ ICAM-1 and VCAM-1 release, ↔ MCP-1, ↓ cell migration and adhesion	Giménez-Bastida et al., 2012 [28]
	Caco-2	Pomegranate husk extract (containing 8.1 μM PUNI and 7.9 μM EA) or PUNI (50 μM) for 1 h as pre-treatment and 24 h in concomitant exposure with pro-inflammatory stimulus	basolateral side: IL-1β (25 μg/L) + TNF-α (50 μg/L) + IFN-γ (50 μg/L); apical side: LPS (1 mg/L)	↓ IL-6 and MCP-1transcription, ↔ IL-8 transcription, ↓ IL-6, IL-8, and MCP-1 secretion	Hollebeeck et al., 2012 [29]
Immune cells	KU812	POMx^{TM} extract (20, 40, and 100 μg/mL) for 2 h prior to pro-inflammatory stimulus	PMA (40 nM) + A23187 (1 μM)	↓ IL-6 and IL-8 transcription, ↓ IL-6 and IL-8 secretion, ↓ JNK and ERK phosphorylation, ↓ NF-κB activation	Rasheed et al., 2009 [31]
	Primary HGE	EA (12.5, 25, 50, and 100 μM) for 18 h	-	↓ IL-8 transcription ([EA] ≥ 25 μM), ↑ BD2 transcription ([EA] ≥ 25 μM), ↑ SLPI transcription, ↓ CCL20 transcription ([EA] ≥ 50 μM), ↓ CXCL5 transcription ([EA] ≥ 50 μM), ↔ IL-1β secretion, ↑ IL-2 secretion ([EA] = 12.5 μM), ↓ IL-2 secretion ([EA] = 50 μM), ↔ IL-4, IL-6, and TNF-α secretion, ↓ IL-8 secretion ([EA] = 50 μM), ↔ MCP-1 secretion, ↑ CCL5 secretion ([EA] ≥ 12.5 μM), ↑ BD2 secretion ([EA] = 100 μM), ↔ SLPI secretion	Promsong et al., 2015 [30]
Neuronal cells	PC12	Pulp aqueous extract (6.25–800 μg/mL), pulp hydro-alcoholic extract (6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg/mL), PJ extract (6.25, 12.5, 25, 50, 100, 200, 400, and 800 μg/mL) for 2 h prior glucose deprivation	Serum glucose deprivation	↓ DNA damage ([PJ] ≥ 400 μg/mL)	Forouzanfar et al., 2013 [61]
	BV-2	Pomegranate seed oil (25 μg/mL) for 24 h	LPS (1 mg/mL)	↓ NO production, ↓ TNF-α release, ↓ iNOS induction, ↓ caspase 3 activation	Račková et al., 2014 [62]

Table 5. *Cont.*

Cell Model	Primary Cell/Cell Line	Tested Compound(s), Dose, Duration	Pro-Inflammatory Treatment	Biological Effects	Reference
	SK-N-SH	PJ extract (25, 50, 100, and 200 μg/mL) for 24 h	IL-1β (10 U/mL)	↓ PGE_2 release, ↓ COX-2 protein expression, ↓ BACE-1 ([PJ] ≥ 50 μg/mL), ↓ amyloid-β ([PJ] ≥ 100 μg/mL), ↓ IκBα phosphorylation ([PJ] ≥ 50 μg/mL)	Velagapudi et al., 2016 [63]
	Primary astrocytes and BV-2	PUNI (10, 20, and 50 μM) for 1 h	LPS (1 mg/mL)	↓ iNOS and COX-2 protein expression, ↓ APP and BACE-1 protein expression, ↓ IκBα phosphorylation	Kim et al., 2017 [53]
Rheumatoid arthritis cells	MH7A	Delphinidin (10 and 30 μM) for 24 h or 2 h (for ELISA)	TNF-α (20 ng/mL)	↓ IL-1β and IL-6 expression, ↓ COX-2 expression, ↓ p65 acetylation, ↓ NF-κB DNA binding activity	Seong et al., 2011 [66]
Cancer cells	DU145 and PC3	PJ (1% or 5%) for 18 h	-	↓ IL-6 and IL-12 secretion, ↓ IL-1β secretion (DU145 only), ↓ CCL5 secretion (PC3 only)	Wang et al., 2011 [70]

↑: increase; ↔ : no change; ↓: decrease; APP: amyloid precursor protein; BACE-1: β-secretase 1; BD2: β-defensin-2; BV-2: murine microglial cell line; Caco-2: human colorectal adenocarcinoma cell line; CCD18-Co: human colon cell line; CCL5: chemokine (C-C motif) ligand 5; CCL20 chemokine (C-C motif) ligand 20; COX-2: cyclooxygenase-2; CXCL5: chemokine (C-X-C motif) ligand 5; DU145: human prostate carcinoma cell line; EA: ellagic acid; ELISA: enzyme-linked immunosorbent assay; ERK: extracellular signal–regulated kinase; h: hour(s); ICAM-1: intracellular adhesion molecule-1; IFN-γ: interferon γ; IL-1β: interleukin 1β; IL-2: interleukin 2; IL-4: interleukin 4; IL-6: interleukin 6; IL-8: interleukin 8; iNOS: inducible nitric oxide synthase; IκB: inhibitor of NF-κB; JNK: c-Jun N-terminal kinase; KU812: human basophilic leukaemia cell line; LPS: lipopolysaccharide; MCP-1: monocyte chemoattractant protein-1; MH7A: human rheumatoid arthritis synovial cell line; NF-κB: nuclear factor κ light-chain-enhancer of activated B cells; NO: nitric oxide; P65: transcription factor p65; PAI-1: plasminogen activator inhibitor-1; PC3: human prostate cancer cell line; PC12: rat adrenal gland cell line; PGE_2: prostaglandin E_2; PJ: pomegranate juice; PMA: phorbol 12-myristate 13-acetate; PUNI: punicalagin; SK-N-SH: human neuroblastoma cell line; SLPI: secretory leukocyte protease inhibitor; TNF-α: tumour necrosis factor α; Uro-A: urolithin A; Uro-B: urolithin B; VCAM-1: vascular cell adhesion molecule-1.

4. Conclusions and Future Directions

Despite abundant literature on the putative effects of pomegranate fruit or extract on CID and other inflammation-related diseases, a definitive relationship between the consumption of pomegranate products and its beneficial properties has not yet been established. It is likely that the effects are due to the ingestion of pomegranate's bioactive polyphenolic molecules. To date, most scientific research on the promising health benefits of pomegranate have been carried out in animal or cell culture models. Clinical trials are currently being conducted to examine a wide range of the potential health effects of pomegranate. However, these trials are few. The most promising properties of pomegranate thus far are related to its effects on diabetes, metabolic syndrome, and cardiovascular diseases. Further studies are required to determine the specific effects of ET-containing foods [80], and to explain the health benefits of pomegranate on CID.

Acknowledgments: This study was supported by the Italian Ministry of Education, University and Research MIUR – SIR Programme (No. RBSI14LHMB, funded to F.D.) and the Auckland Division, Cancer Society of New Zealand (funding to L.R.F.).

Author Contributions: F.D. and L.R.F. performed the research, analysed the data, and F.D. wrote the first draft of the paper. Both authors edited and contributed to drafts of the manuscript, and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nasef, N.A.; Mehta, S.; Ferguson, L.R. Susceptibility to chronic inflammation: An update. *Arch. Toxicol.* **2017**, *91*, 1131–1141. [CrossRef] [PubMed]
2. Calder, P.C. Inflammation: An introduction. In *Nutrition and Physical Activity in Inflammatory Diseases*; Garg, M., Wood, L.G., Eds.; CABI: Wallingford, UK, 2013; pp. 1–22.
3. Calder, P.C.; Albers, R.; Antoine, J.M.; Blum, S.; Bourdet-Sicard, R.; Ferns, G.A.; Folkerts, G.; Friedmann, P.S.; Frost, G.S.; Guarner, F.; et al. Inflammatory disease processes and interactions with nutrition. *Br. J. Nutr.* **2009**, *101*, S1–S45. [CrossRef] [PubMed]
4. Landete, J.M. Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. *Food Res. Int.* **2011**, *44*, 1150–1160. [CrossRef]
5. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892. [CrossRef] [PubMed]
6. Sahebkar, A.; Ferri, C.; Giorgini, P.; Bo, S.; Nachtigal, P.; Grassi, D. Effects of pomegranate juice on blood pressure: A systematic review and meta-analysis of randomized controlled trials. *Pharmacol. Res.* **2017**, *115*, 149–161. [CrossRef] [PubMed]
7. Lynn, A.; Hamadeh, H.; Leung, W.C.; Russell, J.M.; Barker, M.E. Effects of pomegranate juice supplementation on pulse wave velocity and blood pressure in healthy young and middle-aged men and women. *Plant Foods Hum. Nutr.* **2012**, *67*, 309–314. [CrossRef] [PubMed]
8. Esmaillzadeh, A.; Tahbaz, F.; Gaieni, I.; Alavi-Majd, H.; Azadbakht, L. Cholesterol-lowering effect of concentrated pomegranate juice consumption in type II diabetic patients with hyperlipidemia. *Int. J. Vitam. Nutr. Res.* **2006**, *76*, 147–151. [CrossRef] [PubMed]
9. Rock, W.; Rosenblat, M.; Miller-Lotan, R.; Levy, A.P.; Elias, M.; Aviram, M. Consumption of wonderful variety pomegranate juice and extract by diabetic patients increases paraoxonase 1 association with high-density lipoprotein and stimulates its catalytic activities. *J. Agric. Food Chem.* **2008**, *56*, 8704–8713. [CrossRef] [PubMed]
10. Gonzalez-Ortiz, M.; Martinez-Abundis, E.; Espinel-Bermudez, M.C.; Perez-Rubio, K.G. Effect of pomegranate juice on insulin secretion and sensitivity in patients with obesity. *Ann. Nutr. Metab.* **2011**, *58*, 220–223. [CrossRef] [PubMed]
11. Al-Muammar, M.N.; Khan, F. Obesity: The preventive role of the pomegranate (*Punica granatum*). *Nutrition* **2012**, *28*, 595–604. [CrossRef] [PubMed]

12. Asgary, S.; Sahebkar, A.; Afshani, M.R.; Keshvari, M.; Haghjooyjavanmard, S.; Rafieian-Kopaei, M. Clinical evaluation of blood pressure lowering, endothelial function improving, hypolipidemic and anti-inflammatory effects of pomegranate juice in hypertensive subjects. *Phytother. Res.* **2014**, *28*, 193–199. [CrossRef] [PubMed]
13. Sumner, M.D.; Elliott-Eller, M.; Weidner, G.; Daubenmier, J.J.; Chew, M.H.; Marlin, R.; Raisin, C.J.; Ornish, D. Effects of pomegranate juice consumption on myocardial perfusion in patients with coronary heart disease. *Am. J. Cardiol.* **2005**, *96*, 810–814. [CrossRef] [PubMed]
14. Sahebkar, A.; Simental-Mendía, L.E.; Giorgini, P.; Ferri, C.; Grassi, D. Lipid profile changes after pomegranate consumption: A systematic review and meta-analysis of randomized controlled trials. *Phytomedicine* **2016**, *23*, 1103–1112. [CrossRef] [PubMed]
15. Sahebkar, A.; Gurban, C.; Serban, A.; Andrica, F.; Serban, M.C. Effects of supplementation with pomegranate juice on plasma C-reactive protein concentrations: A systematic review and meta-analysis of randomized controlled trials. *Phytomedicine* **2016**, *23*, 1095–1102. [CrossRef] [PubMed]
16. Liberati, A.; Altman, D.G.; Tetzlaff, J.; Mulrow, C.; Gøtzsche, P.C.; Ioannidis, J.P.; Clarke, M.; Devereaux, P.J.; Kleijnen, J.; Moher, D. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: Explanation and elaboration. *BMJ* **2009**, *339*. [CrossRef] [PubMed]
17. Bachoual, R.; Talmoudi, W.; Boussetta, T.; Braut, F.; El-Benna, J. An aqueous pomegranate peel extract inhibits neutrophil myeloperoxidase in vitro and attenuates lung inflammation in mice. *Food Chem. Toxicol.* **2011**, *49*, 1224–1228. [CrossRef] [PubMed]
18. De Oliveira, J.F.; Garreto, D.V.; da Silva, M.C.; Fortes, T.S.; de Oliveira, R.B.; Nascimento, F.R.; Da Costa, F.B.; Grisotto, M.A.; Nicolete, R. Therapeutic potential of biodegradable microparticles containing *Punica granatum* L. (pomegranate) in murine model of asthma. *Inflamm. Res.* **2013**, *62*, 971–980. [CrossRef] [PubMed]
19. Husari, A.; Hashem, Y.; Bitar, H.; Dbaibo, G.; Zaatari, G.; El Sabban, M. Antioxidant activity of pomegranate juice reduces emphysematous changes and injury secondary to cigarette smoke in an animal model and human alveolar cells. *Int. J. Chron. Obstruct. Pulmon. Dis.* **2016**, *11*, 227–237. [CrossRef] [PubMed]
20. Boussetta, T.; Raad, H.; Letteron, P.; Gougerot-Pocidalo, M.A.; Marie, J.C.; Driss, F.; El-Benna, J. Punicic acid a conjugated linolenic acid inhibits TNFa-induced neutrophil hyperactivation and protects from experimental colon inflammation in rats. *PLoS ONE* **2009**, *4*, e6458. [CrossRef] [PubMed]
21. Kim, H.; Banerjee, N.; Ivanov, I.; Pfent, C.M.; Prudhomme, K.R.; Bisson, W.H.; Dashwood, R.H.; Talcott, S.T.; Mertens-Talcott, S.U. Comparison of anti-inflammatory mechanisms of mango (*Mangifera Indica* L.) and pomegranate (*Punica Granatum* L.) in a preclinical model of colitis. *Mol. Nutr. Food Res.* **2016**, *60*, 1912–1923. [CrossRef] [PubMed]
22. Larrosa, M.; González-Sarrías, A.; Yáñez-Gascón, M.J.; Selma, M.V.; Azorín-Ortuño, M.; Toti, S.; Tomás-Barberán, F.; Dolara, P.; Espín, J.C. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. *J. Nutr. Biochem.* **2010**, *21*, 717–725. [CrossRef] [PubMed]
23. Marín, M.; Maria Giner, R.; Ríos, J.L.; Recio, M.C. Intestinal anti-inflammatory activity of ellagic acid in the acute and chronic dextrane sulfate sodium models of mice colitis. *J. Ethnopharmacol.* **2013**, *150*, 925–934. [CrossRef] [PubMed]
24. Rosillo, M.A.; Sánchez-Hidalgo, M.; Cárdeno, A.; Aparicio-Soto, M.; Sánchez-Fidalgo, S.; Villegas, I.; de la Lastra, C.A. Dietary supplementation of an ellagic acid-enriched pomegranate extract attenuates chronic colonic inflammation in rats. *Pharmacol. Res.* **2012**, *66*, 235–242. [CrossRef] [PubMed]
25. Rosillo, M.A.; Sánchez-Hidalgo, M.; Cárdeno, A.; de la Lastra, C.A. Protective effect of ellagic acid, a natural polyphenolic compound, in a murine model of Crohn's disease. *Biochem. Pharmacol.* **2011**, *82*, 737–745. [CrossRef] [PubMed]
26. Shah, T.A.; Parikh, M.; Patel, K.V.; Patel, K.G.; Joshi, C.G.; Gandhi, T.R. Evaluation of the effect of *Punica granatum* juice and punicalagin on NFκB modulation in inflammatory bowel disease. *Mol. Cell. Biochem.* **2016**, *419*, 65–74. [CrossRef] [PubMed]
27. Singh, K.; Jaggi, A.S.; Singh, N. Exploring the ameliorative potential of *Punica granatum* in dextran sulfate sodium induced ulcerative colitis in mice. *Phytother. Res.* **2009**, *23*, 1565–1574. [CrossRef] [PubMed]
28. Giménez-Bastida, J.A.; Larrosa, M.; González-Sarrías, A.; Tomás-Barberán, F.; Espín, J.C.; García-Conesa, M.T. Intestinal ellagitannin metabolites ameliorate cytokine-induced inflammation and associated molecular markers in human colon fibroblasts. *J. Agric. Food Chem.* **2012**, *60*, 8866–8876. [CrossRef] [PubMed]

29. Hollebeeck, S.; Winand, J.; Hérent, M.F.; During, A.; Leclercq, J.; Larondelle, Y.; Schneider, Y.J. Anti-inflammatory effects of pomegranate (*Punica granatum* L.) husk ellagitannins in Caco-2 cells, an in vitro model of human intestine. *Food Funct.* **2012**, *3*, 875–885. [CrossRef] [PubMed]
30. Promsong, A.; Chung, W.O.; Satthakarn, S.; Nittayananta, W. Ellagic acid modulates the expression of oral innate immune mediators: Potential role in mucosal protection. *J. Oral Pathol. Med.* **2015**, *44*, 214–221. [CrossRef] [PubMed]
31. Rasheed, Z.; Akhtar, N.; Anbazhagan, A.N.; Ramamurthy, S.; Shukla, M.; Haqqi, T.M. Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP Kinases and NF-κB in human KU812 cells. *J. Inflamm.* **2009**, *6*. [CrossRef] [PubMed]
32. Asghari, G.; Sheikholeslami, S.; Mirmiran, P.; Chary, A.; Hedayati, M.; Shafiee, A.; Azizi, F. Effect of pomegranate seed oil on serum TNF-a level in dyslipidemic patients. *Int. J. Food Sci. Nutr.* **2012**, *63*, 368–371. [CrossRef] [PubMed]
33. Asgary, S.; Keshvari, M.; Sahebkar, A.; Hashemi, M.; Rafieian-Kopaei, M. Clinical investigation of the acute effects of pomegranate juice on blood pressure and endothelial function in hypertensive individuals. *ARYA Atheroscler.* **2013**, *9*, 326–331. [PubMed]
34. Shishehbor, F.; Mohammad Shahi, M.; Zarei, M.; Saki, A.; Zakerkish, M.; Shirani, F.; Zare, M. Effects of concentrated pomegranate juice on subclinical inflammation and cardiometabolic risk factors for type 2 diabetes: A quasi-experimental study. *Int. J. Endocrinol. Metab.* **2016**, *14*, e33835. [CrossRef] [PubMed]
35. Moazzen, H.; Alizadeh, M. Effects of pomegranate juice on cardiovascular risk factors in patients with metabolic syndrome: A double-blinded, randomized crossover controlled trial. *Plant Foods Hum. Nutr.* **2017**, *72*, 126–133. [CrossRef] [PubMed]
36. Shema-Didi, L.; Sela, S.; Ore, L.; Shapiro, G.; Geron, R.; Moshe, G.; Kristal, B. One year of pomegranate juice intake decreases oxidative stress, inflammation, and incidence of infections in hemodialysis patients: A randomized placebo-controlled trial. *Free Radic. Biol. Med.* **2012**, *53*, 297–304. [CrossRef] [PubMed]
37. Rivara, M.B.; Mehrotra, R.; Linke, L.; Ruzinski, J.; Ikizler, T.A.; Himmelfarb, J. A pilot randomized crossover trial assessing the safety and short-term effects of pomegranate supplementation in hemodialysis patients. *J. Ren. Nutr.* **2015**, *25*, 40–49. [CrossRef] [PubMed]
38. Wu, P.T.; Fitschen, P.J.; Kistler, B.M.; Jeong, J.H.; Chung, H.R.; Aviram, M.; Phillips, S.A.; Fernhall, B.; Wilund, K.R. Effects of pomegranate extract supplementation on cardiovascular risk factors and physical function in hemodialysis patients. *J. Med. Food* **2015**, *18*, 941–949. [CrossRef] [PubMed]
39. Al Hariri, M.; Zibara, K.; Farhat, W.; Hashem, Y.; Soudani, N.; Al Ibrahim, F.; Hamade, E.; Zeidan, A.; Husari, A.; Kobeissy, F. Cigarette smoking-induced cardiac hypertrophy, vascular inflammation and injury are attenuated by antioxidant supplementation in an animal model. *Front. Pharmacol.* **2016**, *7*. [CrossRef] [PubMed]
40. Al-Jarallah, A.; Igdoura, F.; Zhang, Y.; Tenedero, C.B.; White, E.J.; MacDonald, M.E.; Igdoura, S.A.; Trigatti, B.L. The effect of pomegranate extract on coronary artery atherosclerosis in SR-BI/APOE double knockout mice. *Atherosclerosis* **2013**, *228*, 80–89. [CrossRef] [PubMed]
41. Betanzos-Cabrera, G.; Guerrero-Solano, J.A.; Martínez-Pérez, M.M.; Calderón-Ramos, Z.G.; Belefant-Miller, H.; Cancino-Diaz, J.C. Pomegranate juice increases levels of paraoxonase1 (PON1) expression and enzymatic activity in streptozotocin-induced diabetic mice fed with a high-fat diet. *Food Res. Int.* **2011**, *44*, 1381–1385. [CrossRef]
42. Çukurova, Z.; Hergünsel, O.; Eren, G.; Gedikbaşi, A.; Uhri, M.; Demir, G.; Tekdöş, Y. The effect of pomegranate juice on diabetes-related oxidative stress in rat lung. *Türkiye Klinikeri J. Med. Sci.* **2012**, *32*, 444–452. [CrossRef]
43. de Nigris, F.; Balestrieri, M.L.; Williams-Ignarro, S.; D'Armiento, F.P.; Fiorito, C.; Ignarro, L.J.; Napoli, C. The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats. *Nitric Oxide* **2007**, *17*, 50–54. [CrossRef] [PubMed]
44. Dushkin, M.; Khrapova, M.; Kovshik, G.; Chasovskikh, M.; Menshchikova, E.; Trufakin, V.; Shurlygina, A.; Vereschagin, E. Effects of *Rhaponticum carthamoides* versus *Glycyrrhiza glabra* and *Punica granatum* extracts on metabolic syndrome signs in rats. *BMC Complement. Altern. Med.* **2014**, *14*. [CrossRef] [PubMed]

45. Harzallah, A.; Hammami, M.; Kępczyńska, M.A.; Hislop, D.C.; Arch, J.R.; Cawthorne, M.A.; Zaibi, M.S. Comparison of potential preventive effects of pomegranate flower, peel and seed oil on insulin resistance and inflammation in high-fat and high-sucrose diet-induced obesity mice model. *Arch. Physiol. Biochem.* **2016**, *122*, 75–87. [CrossRef] [PubMed]
46. Hontecillas, R.; O'Shea, M.; Einerhand, A.; Diguardo, M.; Bassaganya-Riera, J. Activation of PPAR g and a by punicic acid ameliorates glucose tolerance and suppresses obesity-related inflammation. *J. Am. Coll. Nutr.* **2009**, *28*, 184–195. [CrossRef] [PubMed]
47. Neyrinck, A.M.; Van Hée, V.F.; Bindels, L.B.; De Backer, F.; Cani, P.D.; Delzenne, N.M. Polyphenol-rich extract of pomegranate peel alleviates tissue inflammation and hypercholesterolaemia in high-fat diet-induced obese mice: Potential implication of the gut microbiota. *Br. J. Nutr.* **2013**, *109*, 802–809. [CrossRef] [PubMed]
48. Noori, M.; Jafari, B.; Hekmatdoost, A. Pomegranate juice prevents development of non-alcoholic fatty liver disease in rats by attenuating oxidative stress and inflammation. *J. Sci. Food Agric.* **2017**, *97*, 2327–2332. [CrossRef] [PubMed]
49. Panchal, S.K.; Ward, L.; Brown, L. Ellagic acid attenuates high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. *Eur. J. Nutr.* **2013**, *52*, 559–568. [CrossRef] [PubMed]
50. Taheri Rouhi, S.Z.; Sarker, M.M.; Rahmat, A.; Alkahtani, S.A.; Othman, F. The effect of pomegranate fresh juice versus pomegranate seed powder on metabolic indices, lipid profile, inflammatory biomarkers, and the histopathology of pancreatic islets of Langerhans in streptozotocin-nicotinamide induced type 2 diabetic Sprague-Dawley rats. *BMC Complement. Altern. Med.* **2017**, *17*. [CrossRef]
51. Vilahur, G.; Padró, T.; Casani, L.; Mendieta, G.; López, J.A.; Streitenberger, S.; Badimon, L. Polyphenol-enriched diet prevents coronary endothelial dysfunction by activating the Akt/eNOS pathway. *Rev. Esp. Cardiol.* **2015**, *68*, 216–225. [CrossRef] [PubMed]
52. Zou, X.; Yan, C.; Shi, Y.; Cao, K.; Xu, J.; Wang, X.; Chen, C.; Luo, C.; Li, Y.; Gao, J.; et al. Mitochondrial dysfunction in obesity-associated nonalcoholic fatty liver disease: The protective effects of pomegranate with its active component punicalagin. *Antioxid. Redox Signal.* **2014**, *21*, 1557–1570. [CrossRef] [PubMed]
53. Kim, Y.E.; Hwang, C.J.; Lee, H.P.; Kim, C.S.; Son, D.J.; Ham, Y.W.; Hellström, M.; Han, S.B.; Kim, H.S.; Park, E.K.; et al. Inhibitory effect of punicalagin on lipopolysaccharide-induced neuroinflammation, oxidative stress and memory impairment via inhibition of nuclear factor-κB. *Neuropharmacology* **2017**, *117*, 21–32. [CrossRef] [PubMed]
54. Binyamin, O.; Larush, L.; Frid, K.; Keller, G.; Friedman-Levi, Y.; Ovadia, H.; Abramsky, O.; Magdassi, S.; Gabizon, R. Treatment of a multiple sclerosis animal model by a novel nanodrop formulation of a natural antioxidant. *Int. J. Nanomed.* **2015**, *10*, 7165–7174.
55. Braidy, N.; Essa, M.M.; Poljak, A.; Selvaraju, S.; Al-Adawi, S.; Manivasagm, T.; Thenmozhi, A.J.; Ooi, L.; Sachdev, P.; Guillemin, G.J. Consumption of pomegranates improves synaptic function in a transgenic mice model of Alzheimer's disease. *Oncotarget* **2016**, *7*, 64589–64604. [CrossRef] [PubMed]
56. Essa, M.M.; Subash, S.; Akbar, M.; Al-Adawi, S.; Guillemin, G.J. Long-term dietary supplementation of pomegranates, figs and dates alleviate neuroinflammation in a transgenic mouse model of Alzheimer's disease. *PLoS ONE* **2015**, *10*, e0120964. [CrossRef] [PubMed]
57. Mansouri, M.T.; Farbood, Y.; Naghizadeh, B.; Shabani, S.; Mirshekar, M.A.; Sarkaki, A. Beneficial effects of ellagic acid against animal models of scopolamine- and diazepam-induced cognitive impairments. *Pharm. Biol.* **2016**, *54*, 1947–1953. [CrossRef] [PubMed]
58. Morzelle, M.C.; Salgado, J.M.; Telles, M.; Mourelle, D.; Bachiega, P.; Buck, H.S.; Viel, T.A. Neuroprotective effects of pomegranate peel extract after chronic infusion with amyloid-b peptide in mice. *PLoS ONE* **2016**, *11*, e0166123. [CrossRef] [PubMed]
59. Rojanathammanee, L.; Puig, K.L.; Combs, C.K. Pomegranate polyphenols and extract inhibit nuclear factor of activated T-cell activity and microglial activation in vitro and in a transgenic mouse model of Alzheimer disease. *J. Nutr.* **2013**, *143*, 597–605. [CrossRef] [PubMed]
60. Tapias, V.; Cannon, J.R.; Greenamyre, J.T. Pomegranate juice exacerbates oxidative stress and nigrostriatal degeneration in Parkinson's disease. *Neurobiol. Aging* **2014**, *35*, 1162–1176. [CrossRef] [PubMed]
61. Forouzanfar, F.; Afkhami Goli, A.; Asadpour, E.; Ghorbani, A.; Sadeghnia, H.R. Protective effect of *Punica granatum* L. against serum/glucose deprivation-induced PC12 cells injury. *Evid. Based Complement. Altern. Med.* **2013**, *2013*. [CrossRef] [PubMed]

62. Račková, L.; Ergin, V.; Burcu Bali, E.; Kuniaková, M.; Karasu, Ç. Pomegranate seed oil modulates functions and survival of BV-2 microglial cells in vitro. *Int. J. Vitam. Nutr. Res.* **2014**, *84*, 295–309. [CrossRef] [PubMed]
63. Velagapudi, R.; Baco, G.; Khela, S.; Okorji, U.; Olajide, O. Pomegranate inhibits neuroinflammation and amyloidogenesis in IL-1b-stimulated SK-N-SH cells. *Eur. J. Nutr.* **2016**, *55*, 1653–1660. [CrossRef] [PubMed]
64. Ghavipour, M.; Sotoudeh, G.; Tavakoli, E.; Mowla, K.; Hasanzadeh, J.; Mazloom, Z. Pomegranate extract alleviates disease activity and some blood biomarkers of inflammation and oxidative stress in Rheumatoid Arthritis patients. *Eur. J. Clin. Nutr.* **2017**, *71*, 92–96. [CrossRef] [PubMed]
65. Shukla, M.; Gupta, K.; Rasheed, Z.; Khan, K.A.; Haqqi, T.M. Consumption of hydrolyzable tannins-rich pomegranate extract suppresses inflammation and joint damage in rheumatoid arthritis. *Nutrition* **2008**, *24*, 733–743. [CrossRef] [PubMed]
66. Seong, A.R.; Yoo, J.Y.; Choi, K.; Lee, M.H.; Lee, Y.H.; Lee, J.; Jun, W.; Kim, S.; Yoon, H.G. Delphinidin, a specific inhibitor of histone acetyltransferase, suppresses inflammatory signaling via prevention of NF-κB acetylation in fibroblast-like synoviocyte MH7A cells. *Biochem. Biophys. Res. Commun.* **2011**, *410*, 581–586. [CrossRef] [PubMed]
67. Bhatia, D.; Thoppil, R.J.; Mandal, A.; Samtani, K.A.; Darvesh, A.S.; Bishayee, A. Pomegranate bioactive constituents suppress cell proliferation and induce apoptosis in an experimental model of hepatocellular carcinoma: Role of Wnt/b-catenin signaling pathway. *Evid. Based Complement. Altern. Med.* **2013**, *2013*. [CrossRef] [PubMed]
68. Ammar, A.E.; Esmat, A.; Hassona, M.D.; Tadros, M.G.; Abdel-Naim, A.B.; Guns, E.S. The effect of pomegranate fruit extract on testosterone-induced BPH in rats. *Prostate* **2015**, *75*, 679–692. [CrossRef] [PubMed]
69. Malik, A.; Mukhtar, H. Prostate cancer prevention through pomegranate fruit. *Cell Cycle* **2006**, *5*, 371–373. [PubMed]
70. Wang, L.; Alcon, A.; Yuan, H.; Ho, J.; Li, Q.J.; Martins-Green, M. Cellular and molecular mechanisms of pomegranate juice-induced anti-metastatic effect on prostate cancer cells. *Integr. Biol.* **2011**, *3*, 742–754. [CrossRef] [PubMed]
71. Kojadinovic, M.I.; Arsic, A.C.; Debeljak-Martacic, J.D.; Konic-Ristic, A.I.; Kardum, N.D.; Popovic, T.B.; Glibetic, M.D. Consumption of pomegranate juice decreases blood lipid peroxidation and levels of arachidonic acid in women with metabolic syndrome. *J. Sci. Food Agric.* **2017**, *97*, 1798–1804. [CrossRef] [PubMed]
72. Clinical Tricals.gov. Available online: http://www.clinicaltrials.gov (accessed on 20 February 2017).
73. Kaulmann, A.; Bohn, T. Bioactivity of polyphenols: Preventive and adjuvant strategies toward reducing inflammatory bowel diseases-promises, perspectives, and pitfalls. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 9346470. [CrossRef] [PubMed]
74. Labsi, M.; Khelifi, L.; Mezioug, D.; Soufli, I.; Touil-Boukoffa, C. Antihydatic and immunomodulatory effects of *Punica granatum* peel aqueous extract in a murine model of echinococcosis. *Asian Pac. J. Trop. Med.* **2016**, *9*, 211–220. [CrossRef] [PubMed]
75. Pantuck, A.J.; Leppert, J.T.; Zomorodian, N.; Aronson, W.; Hong, J.; Barnard, R.J.; Seeram, N.; Liker, H.; Wang, H.; Elashoff, R.; et al. Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer. *Clin. Cancer Res.* **2006**, *12*, 4018–4026. [CrossRef] [PubMed]
76. Paller, C.J.; Ye, X.; Wozniak, P.J.; Gillespie, B.K.; Sieber, P.R.; Greengold, R.H.; Stockton, B.R.; Hertzman, B.L.; Efros, M.D.; Roper, R.P.; et al. A randomized phase II study of pomegranate extract for men with rising PSA following initial therapy for localized prostate cancer. *Prostate Cancer Prostatic Dis.* **2013**, *16*, 50–55. [CrossRef] [PubMed]
77. Danesi, F.; Kroon, P.A.; Saha, S.; de Biase, D.; D'Antuono, L.F.; Bordoni, A. Mixed pro- and anti-oxidative effects of pomegranate polyphenols in cultured cells. *Int. J. Mol. Sci.* **2014**, *15*, 19458–19471. [CrossRef] [PubMed]
78. Aragonès, G.; Danesi, F.; Del Rio, D.; Mena, P. The importance of studying cell metabolism when testing the bioactivity of phenolic compounds. *Trends Food Sci. Technol.* **2017**. [CrossRef]

79. Danesi, F.; Philpott, M.; Huebner, C.; Bordoni, A.; Ferguson, L.R. Food-derived bioactives as potential regulators of the IL-12/IL-23 pathway implicated in inflammatory bowel diseases. *Mutat. Res.* **2010**, *690*, 139–144. [CrossRef] [PubMed]
80. González-Sarrías, A.; García-Villalba, R.; Romo-Vaquero, M.; Alasalvar, C.; Örem, A.; Zafrilla, P.; Tomás-Barberán, F.A.; Selma, M.V.; Espín, J.C. Clustering according to urolithin metabotype explains the interindividual variability in the improvement of cardiovascular risk biomarkers in overweight-obese individuals consuming pomegranate: A randomised clinical trial. *Mol. Nutr. Food Res.* **2017**, *61*. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Communication

Polymethoxyflavones: Novel β-Secretase (BACE1) Inhibitors from Citrus Peels

Kumju Youn [1], Yoonjin Yu [1], Jinhyuk Lee [2,3], Woo-Sik Jeong [4], Chi-Tang Ho [5] and Mira Jun [1,*]

1. Department of Food Science and Nutrition, Dong-A University, 37, Nakdong-daero 550 beon-gil, Saha-gu, Busan 49315, Korea; kjyoun@dau.ac.kr (K.Y.); yoonjiinyou@daum.net (Y.Y.)
2. Korean Bioinformation Center, Korea Research Institute of Bioscience and Biotechnology, 125, Gwahak-ro, Yuseong-gu, Daejeon 34141, Korea; mack97hyuk@gmail.com
3. Department of Bioinformatics, University of Sciences and Technology, 100, Hyecheon-ro, Seo-gu, Daejeon 34141, Korea
4. Department of Food & Life Science, College of Biomedical Science & Engineering, Inje University, 197, Inje-ro, Gimhae-si, Gyeongsangnam-do 50834, Korea; jeongws@inje.ac.kr
5. Department of Food Science, Rutgers University, New Brunswick, NJ 08901, USA; ho@aesop.rutgers.edu

* Correspondence: mjun@dau.ac.kr; Tel.: +82-51-200-7323

Received: 31 July 2017; Accepted: 1 September 2017; Published: 4 September 2017

Abstract: Beta-site amyloid precursor protein (APP) cleaving enzyme1 (BACE1) catalyzes the rate-limiting step of amyloid-β protein (Aβ) generation, and is considered as a prime target for Alzheimer's disease (AD). In search of a candidate for AD prevention, our efforts exploring the natural BACE1 inhibitor have led to the finding of nobiletin, tangeretin, and sinensetin—representative compounds of polymethoxyflavones (PMFs). Tangeretin exhibited the strongest BACE1 inhibition (IC_{50}, 4.9×10^{-5} M), followed by nobiletin and sinensetin with IC_{50} values of 5.9×10^{-5} M and 6.3×10^{-5} M, respectively. In addition, all compounds reacted in a non-competitive manner with the substrate. Docking analysis results for complexes with BACE1 indicated that SER10 and THR232 residues of BACE1 hydrogen bonded with two oxygen atoms of tangeretin, while three additional BACE1 residues (ALA157, VAL336 and THR232) interacted with three oxygen atoms of nobiletin. Furthermore, sinensetin formed four hydrogen bonds through nitrogen atoms of TYR71, LYS75, and TRP76, and an oxygen atom of TYR198. Furthermore, the lowest-energy conformations of the most proposed complexes of sinensetin, nobiletin, and tangeretin with BACE1 were −7.2, −7.0, and −6.8 kcal/mol, respectively. Taken together, our results suggest that these polymethoxyflavones (PMFs) might be considered as promising BACE1 inhibitory agents that could lower Aβ production in AD.

Keywords: Alzheimer's disease (AD); β-secretase (BACE1); citrus peel; polymethoxyflavones (PMFs)

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder that alters the mental capacity of patients suffering from the disease [1]. Pathologically, AD occurs mainly because of the generation of two hallmark lesions—neurofibrillary tangles and amyloid plaques—in the brain. Neurofibrillary tangles are insoluble bundles of fibers which are usually made up of phosphorylated tau protein [2]. Amyloid plaques are spherical lesions that contain extracellular aggregates of amyloid-β protein (Aβ). The role of Aβ peptides in the pathogenesis of AD remains unclear; however, several pieces of evidence suggest that an abnormal accumulation of Aβ peptides in the brain is the main cause of AD. Aβ is generated from the amyloid precursor protein (APP) through a two-step proteolytic cleavage. β-Secretase (BACE1) facilitates the first proteolytic step, which releases an N-terminus (sAPPβ) into the extracellular medium. Following β-secretase cleavage, the remaining C99 undergoes

further proteolytic cleavage by γ-secretase to generate the C-terminus of Aβ, and the mature peptide is secreted from the cell [3,4]. Therefore, a strategy to delay or prevent AD could be to stop the proliferation of plaques by inhibiting these secretases.

γ-Secretase is a membrane protein complex composed of presenilin 1 or 2 (PS1 or PS2) as the catalytic subunit, Aph-1a or -b, nicastrin (NCT), and presenilin enhancer-2 (PEN-2). PS1 and PS2 are two integral membrane proteins found in the endoplasmic reticulum and Golgi apparatus, and are the key targets for γ-secretase inhibition in the treatment of AD [5]. However, apart from their essential role in generating the Aβ peptide, PS1 and PS2 regulate the Notch signaling pathway responsible for embryonic development. In addition, only a few types of PS1/PS2-null mouse models survive after birth, because PS1/PS2 knockout mice have substantial neuronal deficits, skeletal defects, underdeveloped subventricular areas, and severe hemorrhages [6–8].

BACE1 knockout mice were initially reported to be free of negative phenotypes; subsequent investigations identified BACE1 null abnormalities such as axon guidance defects, hypomyelination, memory deficits, spin density reduction, impairment of synaptic plasticity, neurogenesis and astrogenesis abnormalities, etc., suggesting that BACE1 inhibitors might produce mechanism-based side-effects [9]. However, the risk of BACE1 mechanism-based toxic effects might depend on the level of BACE1 inhibition. A previous study of BACE1 heterozygote mice overexpressing mutant human APP platelet-derived growth factor promoter (PDAPP;BACE1+/−) showed a considerable reduction in brain Aβ levels and plaque load without side effect [10]. A further study used heterozygous BACE1 gene knockout (BACE1+/−) mice to demonstrate that a 50% BACE1 reduction is sufficient to rescue deficits in brain function without any abnormal effect in an AD transgenic mouse model [11]. Experimental observation from mouse models of AD indicate that a level of BACE1 inhibition between 50% and 75% could be sufficient to reduce the rate of Aβ and prevent amyloid deposition without side effect. Clinical programs today target intermediate steady-state inhibition levels such as 50% or 85% BACE1 inhibition [12]. Therefore, potential mechanism-based side effects might occur only in completely abolishing BACE1 activity, but not in partially inhibiting it. Based on these findings, despite these cautionary notes, the inhibition of BACE1 activity could be a promising molecular target for lowering Aβ in AD.

Recently, much attention has been paid to the screening of products from natural sources, because they are usually considered to be less toxic and have fewer side effects than products from synthetic sources. In particular, the wide biochemical functions of polymethoxyflavones (PMFs) have been studied extensively. They are of particular interest because of their broad range of biological activities, including antioxidant, anticarcinogenic, and anti-inflammatory properties [13–15]. PMFs are found almost exclusively in plants of the genus *Citrus*, and are particularly more abundant in the peel than in other edible parts of the fruit [16]. The neuroprotective properties of citrus peel extract (CPE) have been demonstrated in several studies. CPE induced mild mitochondrial depolarization by inhibiting mitochondrial calcium overload in H_2O_2-stimulated HT-22 neurons [17]. In addition, CPE facilitated cyclic adenosine monophosphate/protein kinase A/extracellular signal-regulated kinase/cAMP response element binding (cAMP/PKA/ERK/CREB) signaling associated with learning and memory in cultured hippocampal neurons [18]. Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), tangeretin (5,6,7,8,4′-pentamethoxyflavo-ne), and sinensetin (5,6,7,3′,4′-pentamethoxyflavone) are the most common PMFs found in citrus peel extract. Although the beneficial effects of the PMFs have been reported previously, the potential of their inhibitory activities against BACE1 in preventing and/or treating AD was first evaluated in this study. In the present study, the activities of nobiletin, tangeretin, and sinensetin as BACE1 inhibitors were assessed, and in silico docking analysis was performed to determine their specific binding sites and lowest binding energies with respect to human BACE1.

2. Materials and Methods

2.1. General

Nobiletin (>98% purity) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tangeretin, sinensetin, (>95% purity), and resveratrol (>99% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). BACE1 inhibition was determined by enzymatic assay using the fluorescent resonance energy transfer (FRET)-based BACE1 kit from Invitrogen (Pan Vera, Madison, WI, USA). α-Secretase (tumor necrosis factor-α converting enzyme, TACE) and substrate were obtained from R&D Systems (Minneapolis, MN, USA). Trypsin, chymotrypsin, elastase, and their substrates were obtained from Sigma-Aldrich (St. Louis, MO, USA). The measurement of fluorescence and optical density was performed with a BioTEK ELISA microplate fluorescence reader FLx 800 and BioTEK ELx 808 (Winooski, VT, USA), respectively.

2.2. Enzymatic Assessment for Biological Evaluation

BACE1, TACE, chymotrypsin, trypsin and elastase assays were performed according to previous methods [19]. Fluorometric BACE1 and TACE assay were measured using a Rh-EVNLDAEFK-Quencher and Mca-PLAQAV-Dpa-RSSSR-NH_2 as substrates. Trypsin, chymotrypsin, and elastase were assayed according to the manual described in the reference using *N*-benzoyl-L-Arg-pNA, *N*-benzoyl-L-Tyr-pNA, and *N*-succinyl-Ala-Ala-Ala-pNA as substrates, respectively.

2.3. Assessment of the Inhibition Kinetics on BACE1

The kinetic mechanisms of the different samples towards BACE1 were determined by the graphical views of Dixon and Lineweaver–Burk plots. The inhibitory constants (Ki) value was defined by interpretation of the Dixon plot, where the value of the x-axis implies -Ki. Maximum velocity (Vmax) and Michaelis constant (Km) were obtained by Lineweaver–Burk plots, using initial velocities obtained over a substrate concentration ranging from 250 to 750 nM. The kinetic parameters were then calculated using Enzyme Kinetic^{TM} module of SigmaPlot^{TM} version 12.3 (Systat Software, Inc., San Jose, CA, USA).

2.4. In Silico Docking Studies

A computational ligand–target docking method was used to investigate structural complexes of the BACE1 (target) with PMFs (ligand) in order to understand the structural basis of this protein target specificity [20]. Specifically, we used Autodock Vina to dock different compounds into the binding pocket residue of the BACE1 crystallographic structure, which was defined as all residues 5 Å from the inhibitor in the original complex. For docking analysis, the crystal structure of the BACE1 protein target was prepared from the protein sequence alignment (Protein Data Bank (PDB ID 2WJO)) and for nobiletin, tangeretin and sinensetin were obtained from the PubChem database (CID 72344, 68077 and 145659, respectively). Chemical structures were drawn and displayed using Marvin (5.11.4, 2012, ChemAxon, One Broadway, Cambridge, MA, USA) [21]. All docking structures were clustered and categorized by the lowest energy and the largest number of clusters.

2.5. Statistical Analysis

All experiments were presented as the mean ± standard deviation (SD) of three independent experiments. Significant differences were conducted by Duncan's multiple range tests using Statistical Analysis System (SAS) version 9.3 (Cary, NC, USA).

3. Results

3.1. In Vitro BACE1 Inhibitory Activity of Biochanin A

The chemical structures of nobiletin, tangeretin, and sinensetin are shown in Figure 1. As shown in Figure 2, the three compounds blocked BACE1 in a dose-dependent manner ($p < 0.001$). Tangeretin had the highest BACE1 inhibitory property (IC_{50}, 4.9×10^{-5} M), followed by nobiletin (IC_{50}, 5.9×10^{-5} M) and sinensetin (IC_{50}, 6.3×10^{-5} M). The common structures of nobiletin, tangeretin, and sinensetin include three methoxy groups at C5, C6, and C7 in the A ring and one methoxy group at C4′ in the B ring, which provide a partial BACE1-suppressive potency. Interestingly, the presence of C3′-OCH_3 in the B ring in nobiletin and sinensetin reduced their inhibitory potency. However, an additional C8-OCH_3 in the A ring of tangeretin noticeably enhanced its anti-BACE1 activity. Therefore, the C8-OCH_3 in the A ring was considered an enhancer of the anti-BACE1 activity, whereas the anti-BACE1 activity decreased in the presence of C3′-OCH_3 in the B ring.

To prove the enzyme specificity of PMFs against BACE1, their inhibitory activities against BACE1 were compared with their inhibitory activities against TACE and other serine proteases (e.g., trypsin, chymotrypsin, and elastase) (Table 1). None of the tested compounds showed statistically significant inhibition against TACE or other serine proteases, suggesting that nobiletin, tangeretin, and sinensetin are specific inhibitors of BACE1.

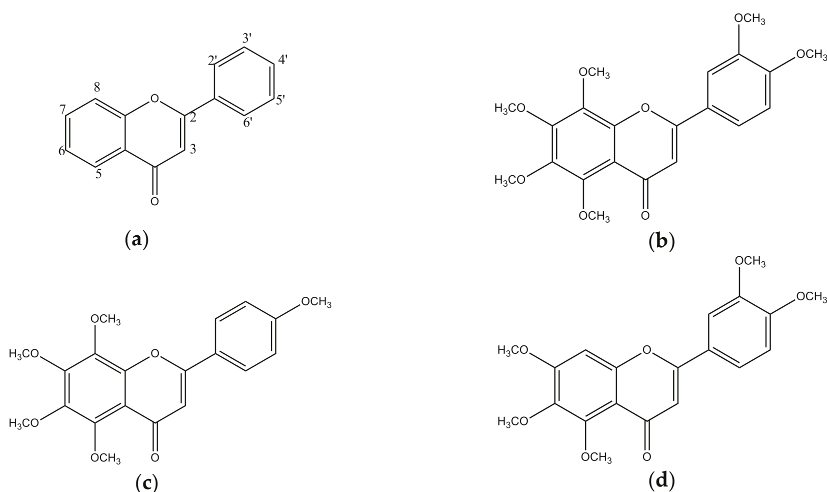


Figure 1. The chemical structures of polymethoxyflavones (PMFs): (**a**) flavone; (**b**) nobiletin; (**c**) tangeretin; (**d**) sinensetin.

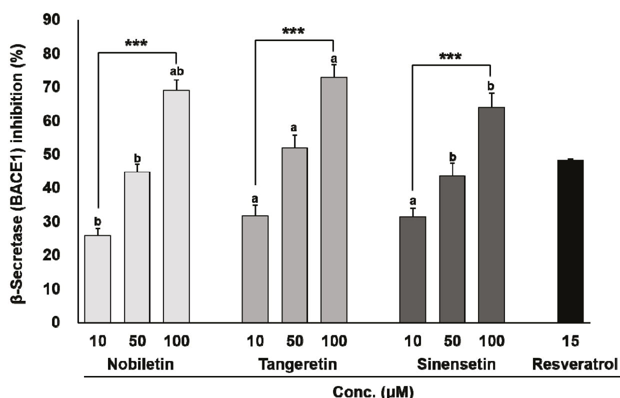


Figure 2. β-Secretase (BACE1) inhibitory activities of polymethoxyflavones (PMFs). The activities (%) are expressed as mean ± standard deviation (SD) of three independent experiments. Each concentration of the same compounds is significantly different at *** $p < 0.001$. The same concentrations of each compound with different letters are significantly different at $p < 0.001$.

Table 1. Inhibitory activities (%) of polymethoxyflavones (PMFs) [1,2] against α-secretase (tumor necrosis factor-α converting enzyme, TACE) and other serine proteases

Sample (μM)	TACE	Trypsin	Chymotrypsin	Elastase
Nobiletin				
50	5.49 ± 0.34	1.00 ± 0.07	1.39 ± 0.17	5.51 ± 1.17
100	11.61 ± 3.07	0.92 ± 0.06	1.77 ± 0.06	4.49 ± 1.00
Tangeretin				
50	8.00 ± 1.00	8.10 ± 0.99	5.68 ± 0.86	8.64 ± 0.65
100	11.28 ± 1.66	11.80 ± 1.04	6.16 ± 0.38	10.58 ± 1.09
Sinensetin				
50	7.88 ± 1.12	5.16 ± 0.69	9.08 ± 0.48	7.40 ± 2.09
100	7.19 ± 1.35	4.70 ± 0.58	8.78 ± 0.80	11.41 ± 1.38

[1] The inhibition (%) of PMFs against TACE, trypsin, chymotrypsin, and elastase is expressed as mean ± SD based on three independent experiments; [2] Comparison of concentration level in PMFs is not significantly different.

3.2. BACE1 Kinetic Assay

In the present study, different graphical analyses were carried out to distinguish the type of inhibition by PMFs. Analysis of kinetic parameters obtained from the Dixon and Lineweaver–Burk plots showed that nobiletin, tangeretin, and sinensetin are noncompetitive inhibitors. These compounds decreased the Vmax of FRET substrate decomposition reaction by BACE1, whereas the Km value remained unchanged in the Lineweaver–Burk plot (Figure 3). As shown by the Dixon plot, a change in the slope and y-intercept of the curve was observed in the presence of an inhibitor, but the x-intercept remained unchanged (Figure 4). The obtained Ki values calculated from the Dixon plots were 3.4×10^{-5} M for nobiletin, 3.7×10^{-5} M for tangeretin, and 3.8×10^{-5} M for sinensetin. A lower Ki value represents a stronger bond between an inhibitor and an enzyme, thus implying greater effectiveness of the inhibitor. Thus, our present results suggest that PMFs could be good BACE1 inhibitors.

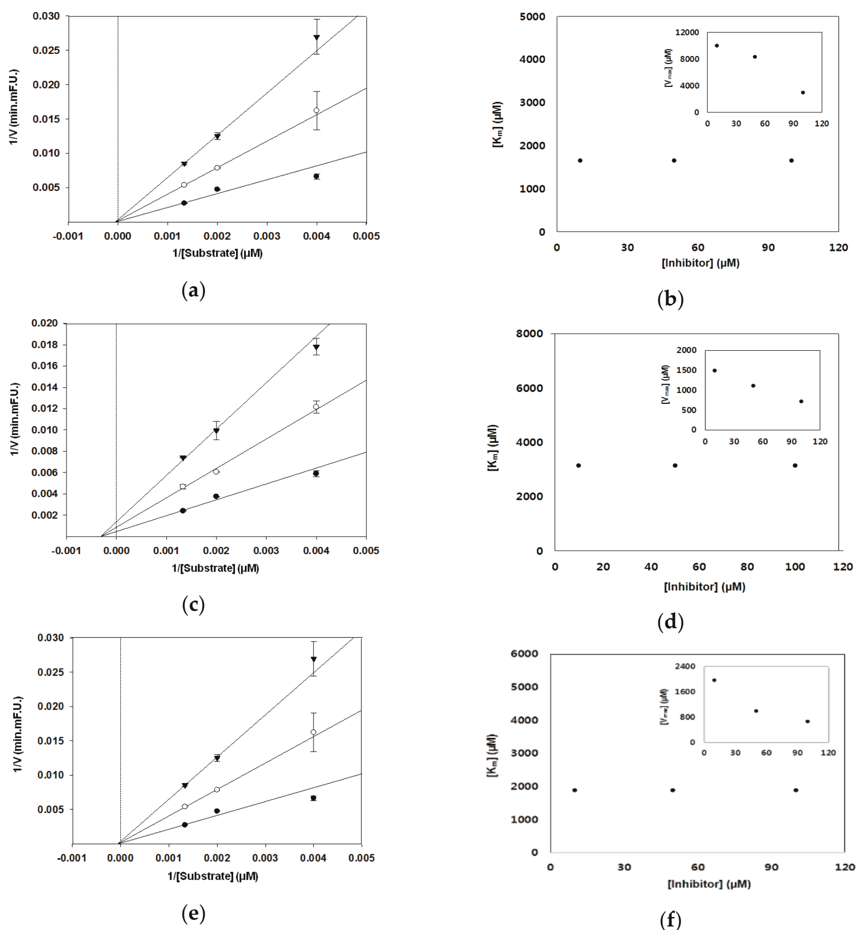


Figure 3. Lineweaver–Burk plot of the reciprocal initial velocities of (**a**) nobiletin, (**c**) tangeretin, and (**e**) sinensetin against BACE1 concentration at different polymethoxyflavone (PMF) concentrations: 10 M (●); 50 μM (○); 100 μM (▼). The Km values as a function of the concentration of (**b**) nobiletin, (**d**) tangeretin, and (**f**) sinensetin. Insets in (**b**,**d**,**f**) show the dependence of the values of Vmax on the concentration of PMFs.

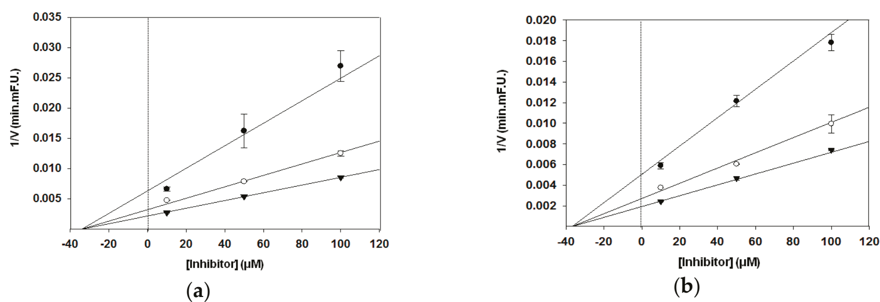
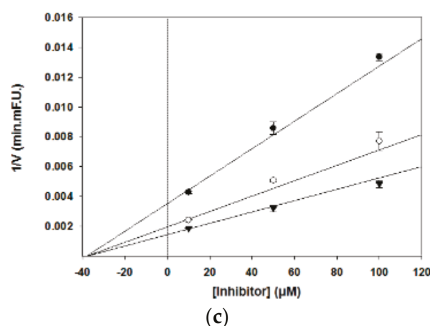


Figure 4. *Cont.*

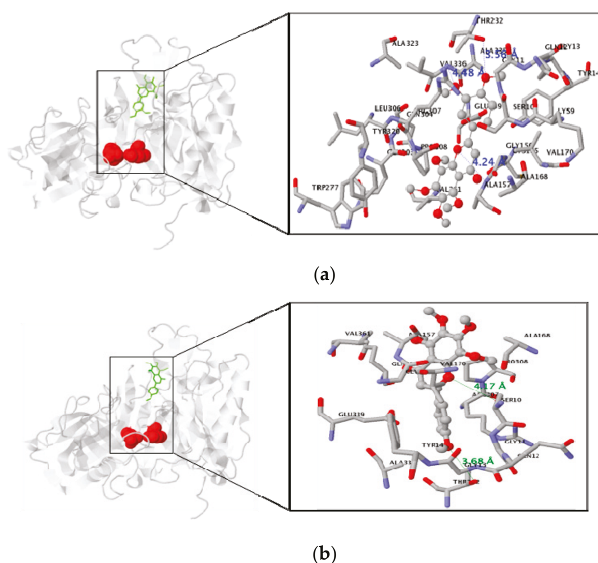


(**c**)

Figure 4. Dixon plot of the reciprocal initial velocities of (**a**) nobiletin, (**b**) tangeretin, and (**c**) sinensetin against BACE1 concentration at several fixed substrate concentrations: 250 nM (●); 500 nM (○); 750 nM (▼).

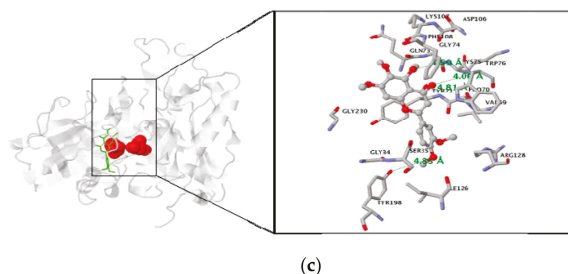
3.3. Molecular Docking Study of the Inhibitory Activity of PMFs against BACE1

The AutoDock program uses a semi-empirical energy force field to guess the binding of protein–ligand complexes of known structure and binding energies. The docking results of the BACE1–PMFs complexes revealed that nobiletin, tangeretin, and sinensetin were stably positioned in allosteric sites of the BACE1 residues, all of which were 5 Å from the inhibitor in the original complex (Figure 5). The oxygen atoms of nobiletin formed three hydrogen bonds with two nitrogen atoms of ALA157 and VAL336 and one oxygen atom of THR232 in BACE1 (distance: 4.24, 4.48, and 3.56 Å, respectively). In comparison, tangeretin had only two hydrogen bonds with oxygen atoms of SER10 and THR232 (distance: 4.17 and 3.68 Å, respectively). Sinensetin formed four hydrogen bonds with nitrogen atoms of TYR71, LYS75, and TRP76, and with an oxygen atom of TYR198 (distance: 4.81, 4.59, 4.06, and 4.86 Å, respectively). In addition, the lowest binding energies of PMFs were negative values: −7 kcal/mol for nobiletin, −6.8 kcal/mol for tangeretin, and −7.2 kcal/mol for sinensetin (Table 2).



(**b**)

Figure 5. *Cont.*



(**c**)

Figure 5. Comparison of in silico docking poses for polymethoxyflavones (PMFs): (**a**) nobiletin, (**b**) tangeretin, (**c**) sinensetin. Representative binding mode of the most stable docking poses of PMFs with BACE1. BACE1 is expressed as a solid ribbon diagram and PMFs as a stick depiction. Left: The complete view of the docking pose of PMFs. Right: The close-up view of the calculated PMFs–BACE1 docking modes. Asp32 and Asp228 are the active catalytic center residues shown in italics. Hydrogen bond interactions between PMFs and the human BACE1 residues are presented as blue (**a**) and green dots (**b**,**c**).

Table 2. Molecular interactions of BACE1 sites with polymethoxyflavones (PMFs).

Ligand	Binding Energy (kcal/mol)	No. of Hydrogen Bonds	Hydrogen Bonds Interacting Residues
Nobiletin	−7.0	3	Residue in 5 Å : ALA157, VAL336, THR232
Tangeretin	−6.8	2	Residue in 5 Å :SER10, THR232
Sinensetin	−7.2	4	Residue in 5 Å : TYR71, LYS75, TRP76, TYR198

4. Discussion

Recently, PMFs were shown to possess neuroprotective effects in both cell and animal models. Nakajima et al. and Nagase et al. reported that the administration of nobiletin for 11 days significantly recovered olfactory bulbectomy-induced memory impairment [22,23]. In addition, APP-SL 7-5 Tg mice were administered nobiletin daily from 4 to 9 months of age, and it significantly reversed memory impairment without affecting general behavior in the context-dependent fear conditioning test and reduced quantity of $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ in the brain [24]. In addition, nobiletin reduced the levels of both soluble $A\beta_{1\text{-}40}$ and reactive oxygen species (ROS) in the brain and in the hippocampus of 3XTg-AD mice [25]. Tangeretin reportedly suppressed LPS-induced primary rat microglia and BV-2 microglial cell activation by modulating the mitogen-activated protein kinase and NF-κB signaling pathways [26]. Subchronic treatment of rats with tangeretin (20 mg/kg/day) for 4 days before 6-oxidopamine (OHDA) injection markedly reduced the loss of both TH+ cells and striatal dopamine content induced by unilateral infusion of 6-OHDA to the medial forebrain bundle [27]. Sinensetin has been studied to a lesser extent than nobiletin and tangeretin, but it was recently shown to activate cyclic AMP response element-mediated transcription in rat hippocampal neurons [18].

Although not fully understood, the metabolites of PMFs have been studied, and it was found that they undergo in vivo biotransformation, producing metabolites with different bioactivities and pharmacological properties. The bioinformation study of nobiletin has shown that the compound undergoes a demethylation pathway with the formation of mono-demethyl nobiletin (DMN) as major metabolites such as 3′-, 4′-, 6-, or 7-DMN. Through di-demethylation, nobiletin transforms to 3′, 4′-di-DMN or 6, 7-di-DMN [28,29]. Like nobiletin, the major nobiletin metabolite 4′-DMN was revealed to stimulate phosphorylation of ERK and CREB signaling pathways related to memory process, and was further shown to be able to cross the blood–brain barrier (BBB) [30]. In addition, 3′-DMN inhibited inducible nitric oxide synthase (iNOS) more efficiently than nobiletin, whereas other two metabolites 4′-DMN and 3′,4′-di-DMN more efficiently suppressed the gene expression of iNOS and cyclooxygenase-2 (COX-2) than their parent compound [31]. As major metabolites of

tangeretin, 4′-demethyltangeretin and 3′,4′-, 7,4′-, or 6,7-demethyltangeretin have been detected [32], and four metabolites of sinensetin (i.e., 4′-, 5-, or 6-OH sinensetin and 7-OH sinensetin sulfate) were identified [33]. Study of the biological actions of the metabolites of both tangeretin and sinensetin are lacking, but it is likely that biological actions similar to nobiletin and its metabolites might also be attributable to these compounds.

Despite the evidence for PMF-mediated neuroprotection, bioavailability in the brain is important for active compounds to be effective in therapeutic for AD, because an inability to cross the blood–brain barrier (BBB) can limit the prevention and/or treatment applications of compounds [34]. When nobiletin was orally administered (50 mg/kg), the content of this compound was revealed to be 3.6 mg/kg (approximately 8.9 μM) in the brains of mice [35]. The previous study also demonstrated nobiletin accumulation in the brain of A/J mice fed nobiletin (250, 500, or 1000 ppm in diet for 16 weeks), which suggested that nobiletin may absorbed and penetrate BBB into the brain tissues where it may directly act as a therapeutic agent on the neural cells. Time-dependent dynamics of nobiletin in the serum and brain showed that nobiletin may rapidly penetrate the BBB and enter the brain tissues, where it may directly act as a therapeutic agent on neural cells, including hippocampal neurons [35]. Interestingly, nobiletin exhibited high accumulation in the brain, which was even much higher than that in the liver [36]. Even though the tested compounds in our study have been revealed to penetrate the BBB and stay active in the brain when orally administered (since they are hydrophobic compounds), orally-consumed PMFs may be poorly absorbed in general. Thus, to overcome the limitation of poor bioavailability of PMFs, several studies have been focused on the formation of amorphous and nanosized nobiletin for enhancing the bioavailability and CNS delivery [37]. Datla and coworkers showed that tangeretin was detected in the hippocampus after chronic oral administration [27]. Furthermore, the multidrug resistance transporter P-glycoprotein (P-gp) is an active component of the BBB, acting as an ATP-driven efflux pump, controlling the movement of structurally diverse molecules across the BBB [38]. Nobiletin and tangeretin have inhibitory potential with P-gp in adriamycin-resistant human myelogenous leukemia cells and in Caco-2 cells using talinolol as a probe [39,40].

Oral acute and chronic toxicity studies are important to determine the safety of drugs and plant products for human use. However, to date, information on the toxicology profile for PMFs is still limited. Ting et al. (2015) [41] reported that rats administered tangeretin up to 1000 to 3000 mg/kg bw showed no evidence of death nor significant change of clinical chemistry in oral acute and sub-acute study. Alterations of the hepatic cell and lipid profile increased dose-dependently and exhibited a distinct injury recovery pattern. The results of a recent pilot clinical study indicated that nobiletin-rich *Citrus reticulata* peel extract treatment for 1 year could prevent the progression of the cognitive impairment in donepezil-preadministered AD patients with no adverse side effects [42].

It is important to reiterate that the risk of mechanism-based toxic effects might depend on the level of BACE1 inhibition. Partial inhibition of BACE1 activity could represent a feasible approach. For example, the currently tested BACE1 inhibitor MK-8931 has been safe and tolerated after multiple-dose administration for at least 18 months in human subjects [12]. Since natural BACE1 inhibitors (e.g., PMFs) have relatively weaker BACE1 inhibitory effects than the synthetic one, they may be free from side effects caused by excessive BACE inhibition. Although further pharmacokinetic explanations of PMFs in an animal model are required, this study provides evidence that PMFs exerted significant and specific inhibitory properties against BACE1.

5. Conclusions

Our findings suggest that PMFs have a significant inhibitory activity against BACE1, whereas they lack any inhibitory property against TACE and other serine proteases. Enzyme kinetics was evaluated using the Dixon and Lineweaver–Burk plots to identify compound inhibition modes. In addition, molecular docking studies indicated strong hydrogen bonding with several important amino acid residues, as evidenced by negative binding energies at the allosteric site in BACE1; this can explain

the potency of these compounds. Although further BACE1 selectivity over cathepsins D and BACE2 and in vivo studies are required to confirm our findings, these PMFs showed significant and selective inhibitory activities against BACE1, and can be used as potential agents for preventing and/or treating AD.

Acknowledgments: This research was supported by Dong-A University.

Author Contributions: Mira Jun designed the study and revised the manuscript and Kumju Youn prepared the manuscript and Yoonjin Yu performed the experiments. Jinhyuk Lee performed molecular docking study, and Woo-Sik Jeong and Chi-Tang Ho analyzed data.

Conflicts of Interest: No conflict of interest exist for any of the authors.

References

1. Selkoe, D.J. Alzheimer's disease results from the cerebral accumulation and cytotoxicity of amyloid β-protein. *J. Alzheimer's Dis.* **2001**, *3*, 75–80. [CrossRef]
2. Ghosh, A.K.; Osswald, H.L. BACE1 (beta-secretase) inhibitors for the treatment of Alzheimer's disease. *Chem. Soc. Rev.* **2014**, *7*, 6765–6813. [CrossRef] [PubMed]
3. Vassar, R.; Kuhn, P.H.; Haass, C.; Kennedy, M.E.; Rajendran, L.; Wong, P.C.; Lichtenthaler, S.F. Function, therapeutic potential and cell biology of BACE proteases: Current status and future prospects. *J. Neurochem.* **2014**, *130*, 4–28. [CrossRef] [PubMed]
4. De Strooper, B.; Annaert, W. Novel research horizons for presenilins and gamma-secretases in cell biology and disease. *Annu. Rev. Cell Dev. Biol.* **2010**, *26*, 235–260. [CrossRef] [PubMed]
5. Seiffert, D.; Bradley, J.D.; Rominger, C.M.; Rominger, D.H.; Yang, F.; Meredith, J.E., Jr.; Wang, Q.; Roach, A.H.; Thompson, L.A.; Spitz, S.M.; et al. Presenilin-1 and -2 are molecular targets for γ-secretase inhibitors. *J. Biol. Chem.* **2000**, *275*, 34086–34091. [CrossRef] [PubMed]
6. Shen, J.; Bronson, R.T.; Chen, D.F.; Xia, W.; Selkoe, D.J.; Tonegawa, S. Skeletal and CNS defects in Presenilin-1-deficient mice. *Cell* **1997**, *89*, 629–639. [CrossRef]
7. Wong, P.C.; Zheng, H.; Chen, H.; Becher, M.W.; Sirinathsinghji, D.J.; Trumbauer, M.E.; Chen, H.Y.; Price, D.L.; Van der Ploeg, L.H.; Sisodia, S.S. Presenilin 1 is required for Notch1 DII1 expression in the paraxial mesoderm. *Nature* **1997**, *387*, 288–292. [CrossRef] [PubMed]
8. Haass, C.; De Strooper, B. The presenilins in Alzheimer's disease-proteolysis holds the key. *Science* **1999**, *286*, 916–919. [CrossRef] [PubMed]
9. Yan, R. Physiological Functions of the β-Site Amyloid Precursor Protein Cleaving Enzyme 1 and 2. *Front. Mol. Neurosci.* **2017**, *10*, 97–106. [CrossRef] [PubMed]
10. McConlogue, L.; Buttini, M.; Anderson, J.P.; Brigham, E.F.; Chen, K.S.; Freedman, S.B.; Games, D.; Johnson-Wood, K.; Lee, M.; Zeller, M.; et al. Partial reduction of BACE1 has dramatic effects on Alzheimer plaque and synaptic pathology in APP Transgenic Mice. *J. Biol. Chem.* **2007**, *282*, 26326–26334. [CrossRef] [PubMed]
11. Kimura, R.; Devi, L.; Ohno, M. Partial reduction of BACE1 improves synaptic plasticity, recent and remote memories in Alzheimer's disease transgenic mice. *J. Nurochem.* **2010**, *113*, 248–261. [CrossRef] [PubMed]
12. Barão, S.; Moechars, D.; Lichtenthaler, S.F.; De Strooper, B. BACE1 Physiological Functions May Limit Its Use as Therapeutic Target for Alzheimer's Disease. *Trends Neurosci.* **2016**, *39*, 158–169. [CrossRef] [PubMed]
13. Parhiz, H.; Roohbakhsh, A.; Soltani, F.; Rezaee, R.; Iranshahi, M. Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: An updated review of their molecular mechanisms and experimental models. *Physiol. Res.* **2015**, *29*, 323–331. [CrossRef] [PubMed]
14. Akao, Y.; Itoh, T.; Ohguchi, K.; Iinuma, M.; Nozawa, Y. Interactive Effects of Polymethoxyflavones from Citrus on Cell Growth Inhibition in Human Neuroblastoma SH-SY5Y cells. *Bioorg. Med. Chem. Lett.* **2008**, *16*, 2803–2810. [CrossRef] [PubMed]
15. Huang, Y.S.; Ho, S.C. Polymethoxyflavones are responsible for the anti-inflammatory activity of citrus fruit peel. *Food Chem.* **2010**, *119*, 868–873. [CrossRef]
16. Manthey, J.A.; Grohmann, K. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. *J. Agric. Food Chem.* **2001**, *49*, 3268–3273. [CrossRef] [PubMed]

17. Wu, T.; Zang, X.; He, M.; Pan, S.; Xu, X. Structure-Activity Relationship of Flavonoids on Their Anti- *Escherichia Coli* Activity and Ihibition of DNA Gyrase. *J. Agric. Food Chem.* **2013**, *61*, 8185–8190. [CrossRef] [PubMed]
18. Kawahata, I.; Yoshida, M.; Sun, W.; Nakajima, A.; Lai, Y.; Osaka, N.; Matsuzaki, K.; Yokosuka, A.; Mimaki, Y.; Naganuma, A.; et al. Potent activity of nobiletin-rich citrus reticulata peel extract to facilitate cAMP/PKA/ERK/CREB signaling associated with learning and memory in cultured hippocampal neurons: Identification of the substances responsible for the pharmacological action. *J. Neural Transm.* **2013**, *120*, 1397–1409. [CrossRef] [PubMed]
19. Youn, K.; Park, J.H.; Lee, J.; Jeong, W.S.; Ho, C.T.; Jun, M. The Identification of Biochanin A as a Potent and Selective β-Site App-Cleaving Enzyme 1 (Bace1) Inhibitor. *Nutrients* **2016**, *8*, 637. [CrossRef] [PubMed]
20. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. [CrossRef] [PubMed]
21. Marvin 5.11.4, 2012, ChemAxon. Available online: http://www.chemaxon.com (accessed on 16 October 2016).
22. Nakajima, A.; Yamakuni, T.; Haraguchi, M.; Omae, N.; Song, S.Y.; Kato, C.; Nakagawasai, O.; Tadano, T.; Yokosuka, A.; Mimaki, Y.; et al. Nobiletin, a citrus flavonoid that improves memory impairment, rescues bulbectomy-induced cholinergic neurodegeneration in mice. *J. Pharmacol. Sci.* **2007**, *105*, 122–126. [CrossRef] [PubMed]
23. Nagase, H.; Omae, N.; Omori, A.; Nakagawasai, O.; Tadano, T.; Yokosuka, A.; Sashida, Y.; Mimaki, Y.; Yamakuni, T.; Ohizumi, Y. Nobiletin and its related flavonoids with CRE-dependent transcription-stimulating and neuritegenic activities. *Biochem. Biophys. Res. Commun.* **2005**, *337*, 1330–1336. [CrossRef] [PubMed]
24. Onozuka, H.; Nakajima, A.; Matsuzaki, K.; Shin, R.W.; Ogino, K.; Saigusa, D.; Tetsu, N.; Yokosuka, A.; Sashida, Y.; Mimaki, Y.; et al. Nobiletin, a citrus flavonoid, improves memory impairment and Abeta pathology in a transgenic mouse model of Alzheimer's disease. *J. Pharmacol. Exp. Ther.* **2008**, *326*, 739744. [CrossRef] [PubMed]
25. Nakajima, A.; Aoyama, Y.; Shin, E.J.; Nam, Y.; Kim, H.C.; Nagai, T.; Yokosuka, A.; Mimaki, Y.; Yokoi, T.; Ohizumi, Y.; et al. Nobiletin, a citrus flavonoid, improves cognitive impairment and reduces soluble Aβ levels in a triple transgenic mouse model of Alzheimer's disease (3XTg-AD). *Behav. Brain. Res.* **2015**, *289*, 69–77. [CrossRef] [PubMed]
26. Shu, Z.P.; Yang, B.Y.; Zhao, H.; Xu, B.Q.; Jiao, W.J.; Wang, Q.H.; Wang, Z.B.; Kuang, H.X. Tangeretin exerts anti-neuroinflammatory effects via NF-κB modulation in lipopolysaccharide-stimulated microglial cells. *Int. Immunopharmacol.* **2014**, *19*, 275–282. [CrossRef] [PubMed]
27. Datla, K.P.; Christidou, M.; Widmer, W.W.; Rooprai, H.K.; Dexter, D.T. Tissue distribution and neuroprotective effects of Citrus flavonoid tangeretin in a rat model of Parkinson's disease. *Neuroreport* **2001**, *12*, 3871–3875. [CrossRef] [PubMed]
28. Murakami, A.; Kuwahara, S.; Takahashi, Y.; Ito, C.; Furukawa, H.; Ju-Ichi, M.; Koshimizu, K.; Ohigashi, H. In vitro absorption and metabolism of nobiletin, a chemopreventive polymethoxyflavonoid in citrus fruits. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 194–197. [CrossRef] [PubMed]
29. Koga, N.; Matsuo, M.; Ohta, C.; Haraguchi, K.; Matsuoka, M.; Kato, Y.; Ishii, T.; Yano, M.; Ohta, H. Comparative study on nobiletin metabolism with liver microsomes from rats, Guinea pigs and hamsters and rat cytochrome p450. *Biol. Pharm. Bull.* **2007**, *12*, 2317–2323. [CrossRef]
30. Al Rahim, M.; Nakajima, A.; Saigusa, D.; Tetsu, N.; Maruyama, Y.; Shibuya, M.; Yamakoshi, H.; Tomioka, Y.; Iwabuchi, Y.; Ohizumi, Y.; et al. 40-Demethylnobiletin, a bioactive metabolite of nobiletin enhancing PKA/ERK/CREB signaling, rescues learning impairment associated with NMDA receptor antagonism via stimulation of the ERK cascade. *Biochemistry* **2009**, *48*, 7713–7721. [CrossRef] [PubMed]
31. Li, S.; Sang, S.; Pan, M.H.; Lai, C.S.; Lo, C.Y.; Yang, C.S.; Ho, C.T. Anti-inflammatory property of the urinary metabolites of nobiletin in mouse. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5177–5181. [CrossRef] [PubMed]
32. Breinholt, V.M.; Rasmussen, S.E.; Brosen, K.; Friedberg, T.H. In vitro metabolism of genistein and tangeretin by human and murine cytochrome P450s. *Pharmacol. Toxicol.* **2003**, *93*, 14–22. [CrossRef] [PubMed]
33. Wei, G.J.; Sheen, J.F.; Lu, W.C.; Hwang, L.S.; Ho, C.T.; Lin, C.I. Identification of sinensetin metabolites in rat urine by an isotope-labeling method and ultrahigh-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Agric. Food Chem.* **2012**, *61*, 5016–5021. [CrossRef] [PubMed]

34. Nakajima, A.; Ohizumi, Y.; Yamada, K. Anti-dementia activity of nobiletin, a citrus flavonoid: A review of animal studies. *Clin. Psycopharmacol. Neurosci.* **2014**, *12*, 75–82. [CrossRef] [PubMed]
35. Saigusa, D.; Shibuya, M.; Jinno, D.; Yamakoshi, H.; Iwabuchi, Y.; Yokosuka, A.; Mimaki, Y.; Naganuma, A.; Ohizumi, Y.; Tomioka, Y.; et al. High-performance liquid chromatography with photodiode array detection for determination of nobiletin content in the brain and serum of mice administrated the natural compound. *Anal. Bioanal. Chem.* **2011**, *400*, 3635–3641. [CrossRef] [PubMed]
36. Wang, M.; Zheng, J.; Zhong, Z.; Song, M.; Wu, X. Tissue distribution of nobiletin and its metabolites in mice after oral administration of nobiletin. *FASEB J.* **2013**, *27* (Suppl. 1), 125.3.
37. Onoue, S.; Uchida, A.; Takahashi, H.; Seto, Y.; Kawabata, Y.; Ogawa, K.; Yuminoki, K.; Hashimoto, N.; Yamada, S. Development of high-energy amorphous solid dispersion of nanosized nobiletin, a citrus polymethoxylated flavone, with improved oral bioavailability. *J. Pharm. Sci.* **2011**, *100*, 3793–3801. [CrossRef] [PubMed]
38. Youdim, K.A.; Dobbie, M.S.; Kuhnle, G.; Proteggente, A.R.; Abbott, N.J.; Rice-Evans, C. Interaction between flavonoids and the blood–brain barrier: In Vitro studies. *J. Neurochem.* **2003**, *85*, 180–192. [CrossRef] [PubMed]
39. Ikegawa, T.; Ushigome, F.; Koyabu, N.; Morimoto, S.; Shoyama, Y.; Naito, M.; Tsuruo, T. Inhibition of P-glycoprotein by orange juice components, polymethoxyflavones in adriamycin-resistant human myelogenous leukemia (K562/ADM) cells. *Cancer Lett.* **2000**, *160*, 21–28. [CrossRef]
40. Mertens-Talcott, S.U.; De Castro, W.V.; Manthey, J.A.; Derendorf, H.; Butterweck, V. Polymethoxylated flavones and other phenolic derivates from citrus in their inhibitory effects on P-glycoprotein-mediated transport of talinolol in Caco-2 cells. *J. Agric. Food Chem.* **2007**, *55*, 2563–2568. [CrossRef] [PubMed]
41. Ting, Y.; Chiou, Y.S.; Jiang, Y.; Pan, M.H.; Lin, Z.; Huang, Q. Safety evaluation of tangeretin and the effect of using emulsion-based delivery system: Oral acute and 28-day sub-acute toxicity study using mice. *Food Res. Int.* **2015**, *74*, 140–150. [CrossRef] [PubMed]
42. Seki, T.; Kamiya, T.; Furukawa, K.; Azumi, M.; Ishizuka, S.; Takayama, S.; Nagase, S.; Arai, H.; Yamakuni, T.; Yaegashi, N. Nobiletin-rich Citrus reticulata peels, a kampo medicine for Alzheimer's disease: A case series. *Geriatr. Gerontol. Int.* **2013**, *13*, 236–238. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Flavonolignans Inhibit IL1-β-Induced Cross-Talk between Blood Platelets and Leukocytes

Michal Bijak [1,*], Angela Dziedzic [1], Ewelina Synowiec [2], Tomasz Sliwinski [2] and Joanna Saluk-Bijak [1]

[1] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland; angela.dziedzic@outlook.com (A.D.); joanna.saluk@biol.uni.lodz.pl (J.S.-B.)

[2] Laboratory Medical Genetics, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland; ewelina.synowiec@biol.uni.lodz.pl (E.S.); tomasz.sliwinski@biol.uni.lodz.pl (T.S.)

* Correspondence: michal.bijak@biol.uni.lodz.pl; Tel./Fax: +48-042-635-4336

Received: 15 August 2017; Accepted: 13 September 2017; Published: 15 September 2017

Abstract: Interleukin-1 beta (IL-1β)—the most potent pro-inflammatory is responsible for a broad spectrum of immune and inflammatory responses, it induces T-cell and B-cell activation and consequently the synthesis of other pro-inflammatory cytokines (such as IFN-γ and TNF). IL-1β induces the formation of blood platelet-leukocyte aggregates (PLAs), which suggests that IL-1β significantly affects the cross-talk between blood platelets and the immune response system, leading to coronary thrombosis. The aim of our study is to investigate the effect of flavonolignans (silybin, silychristin and silydianin) on the IL-1β-induced interaction between platelets and leukocytes, as well as on the expression and the secretion of pro-inflammatory factors. Whole blood samples were pre-incubated with commercially available flavonolignans (silybin, silychristin and silydianin) in a concentration range of 10–100 μM (30 min, 37 °C). Next, samples were activated by IL-1β for 1 h. Blood platelet-leukocyte aggregates were detected by using the double-labeled flow cytometry (CD61/CD45). The level of produced cytokines was estimated via the ELISA immunoenzymatic method. IFN-γ and TNF gene expression was evaluated using Real Time PCR with TaqMan arrays. We observed that in a dose-dependent manner, silybin and silychristin inhibit the IL-1β-induced formation of blood platelet-leukocyte aggregates in whole blood samples, as well as the production of pro-inflammatory cytokines—IL-2, TNF, INF-α, and INF-γ. Additionally, these two flavonolignans abolished the IL-1β-induced expression of mRNA for IFN-γ and TNF. Our current results demonstrate that flavonolignans can be novel compounds used in the prevention of cardiovascular diseases with dual-use action as antiplatelet and anti-inflammatory agents.

Keywords: interleukin 1; anti-inflammatory; flavonolignans; silybin; silychristin

1. Introduction

Interleukin 1 beta (IL-1β) is the most potent pro-inflammatory cytokine that is crucial in host-defense responses to infection and injury [1]. IL-1β is expressed by many cells and has multiple functions, including in local inflammation. IL-1β is produced by activated macrophages, endothelial cells, B cells, and fibroblasts. This potent pro-inflammatory cytokine was initially discovered and classified as the major endogenous pyrogen. IL-1β mediates the expression of a vast array of genes involved in secondary inflammation, which coordinate all aspects of local inflammation and also attract and activate the cells of the adaptive immune system at the infection sites [2]. IL-1β is responsible for a broad spectrum of immune and inflammatory responses, induces T-cell and B-cell activation, and consequently the synthesis of other pro-inflammatory cytokines (such as IFN-γ, IL-6 and

TNF), and antibody production. This cytokine also induces the expression of itself in newly-arriving monocytes, thus reinforcing the overall process. IL-1β circulating in blood is unregulated under systemic and chronic inflammatory conditions and is measurable in pg/mL [3].

The mechanism of IL-1β cell action is based on the binding to type I IL-1 receptor (IL-1RI) and the activation of the intracellular signal pathway. IL-1β first binds to the first extracellular chain of IL-1RI that recruits the IL-1 receptor accessory protein (IL-1RAcP), which serves as a co-receptor and is necessary for signal transduction. In response to the ligand binding of the receptor, a complex sequence of combinatorial phosphorylation and ubiquitination events results in the activation of nuclear factor κB (NF-κB) signalling and the JNK and p38 mitogen-activated protein kinase pathways. Together, these then induce the expression of canonical *IL-1β* target genes through transcriptional and post-transcriptional mechanisms [4].

Pro-inflammatory cytokines and chemokines can affect all of the coagulation pathways. Therefore, the relationship between the presence of cytokines resulting in inflammation and hyper-coagulation state, is particularly relevant in the pathogenesis of thrombosis. Interleukin 1 Receptor 1 and IL-1β have been seen to be increased in cardiovascular diseases [5]. IL-1β is also known to be present in autoimmune conditions and contributes to several chronic diseases, including atherosclerosis [6]. Increased levels of IL-1β are known to play an important role in both acute and chronic inflammation, with resulting pathological clotting. However, there is still little information available about the effects of this interleukin on the properties of blood platelet involved in clot formation. An in vitro study performed using the flow cytometry method indicated that IL-1β significantly increases the formation of blood platelet-leukocyte aggregates (PLAs). This suggests that IL-1β significantly effects the cross-talk between blood platelets and the immune response system [5]. Flavonolignans are a group of active chemical components of silymarin—an extract obtained from the fruit of the milk thistle—*Silybum marianum* (L.) Gaernt. [7]. This plant, which is a member of Asteraceae family, has been used for thousands of years as a remedy for a variety of ailments [8]. Flavonolignans are structurally composed of a flavonoid unit (taxifolin) and a phenylpropanoid unit (coniferyl alcohol), linked by an oxeran ring [9,10]. This type of connection is present in the formation of lignans, and gives this group of compounds its name [11]. Silymarin represents 1.5–3% of the dry fruit weight. The main represents of flavonolignans presented in silymarin are silybin, isosilybin, silychristin, isosilychristin silydianin, silimonin [7,12–16], however the highest concentration, approximately 70% of the extract have the silybin, silychristin and silydianin and these compounds are the major bioactive component of extract [17]. In our previous study, we demonstrated that flavonolignans, especially silybin and silychristin, are able to adenosine diphosphate (ADP)-induce blood platelets' activation through interactions with the P2Y12 receptor [18]. Additionally, silybin and silychristin have an inhibitory effect on platelets cyclooxygenase activity, which blocks arachidonic acid metabolism in these cells [19].

Recent studies demonstrate that the flavonolignans are able to inhibit the NF-κB activation pathway, which is responsible for cell reaction to IL-1β. For this reason, we decided to investigate the effect that flavonolignans (silybin, silychristin and silydianin) have on the IL-1β-induced interaction between platelets and leukocytes, as well as on the expression and secretion of pro-inflammatory and prothrombotic factors.

2. Materials and Methods

2.1. Reagents

Interleukin-1 beta was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Dimethyl sulfoxide (DMSO), Tris and the flavonolignans (silybin, silychristin and silydianin (Figure S1) were all obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Flow cytometry reagents: anti-CD61/FITC, anti-CD61/PE, anti-CD45/PE, isotype controls, BD FACS^{TM} Lysing Solution and CellFix were all obtained from Becton Dickinson (San Diego, CA, USA). All of the other chemicals were of reagent grade or the highest quality available.

2.2. Blood Samples

Blood samples collected from twelve different healthy donors were purchased from the Regional Centre for Transfusion Medicine in Lodz (Poland). All of the samples had been drawn in the morning (between 8 a.m. and 10 a.m.), from fasting donors and immediately transferred to the laboratory. All donors had been checked by a doctor and were found to have had no cardiovascular disorders, allergies, lipid, or carbohydrate metabolism disorders, nor any traces of medication. Blood was collected according to the standard protocol to the CPDA-1 (Citrate Phosphate Dextrose Adenine Solution) containing blood collection bag with double port, 450 mL (KRUUSE, Langeskov, Denmark). Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research, and approved by the Committee on the Ethics of Research in Human Experimentation at the University of Lodz (with Resolution No. 16/KBBN-UŁ/II/2016).

2.3. Samples Preparation

The fresh whole blood samples were pre-incubated with flavonolignans (silybin, silychristin and silydianin) in the concentration range of 10–100 µM, at 37 °C. All of the compounds tested were initially dissolved in 20% DMSO to a preliminary concentration of 20 mM. Other solutions of the compounds used were also 20% DMSO (prepared in 50 mM Tris-buffered saline [TBS], pH 7.4). The final DMSO concentration of all the samples was 0.1%. In the control samples, the same volume of solvent was added (20% DMSO prepared at 50 mM TBS, pH 7.4), with the probes warmed at 37 °C [18–20]. After 30 min, to each sample (control or pre-incubated with flavonolignans) IL-1β (10 ng/mL) was added. Treatment with IL-1β was conducted for 1 h at 37 °C, and samples were used for appropriate analysis. An additional sample was not activated.

2.4. Flow Cytometry Analysis of Platelet-Leukocyte Aggregates

First, the blood samples were stained in BD FACS lysing solution. After 1 h of fixation, the samples were stained with specific antibodies: anti-CD61/FITC, anti-CD45/PE (6 µL of each antibody + 50 µL of sample), and left for 30 min in the dark, at room temperature. Next, 500 µL of 1% Cellfix was added to each sample. All of the samples were centrifuged (2500× *g*, 10 min), and the precipitate obtained was then suspended in 500 µL of 0.9% NaCl. The fluorescence of 10,000 leucocytes (CD45/PE-positive objects) was measured using the CUBE 6 (Pertec, Görlitz, Germany) flow cytometer. Blood platelet-leukocyte aggregates were detected using CD61-FITC and CD45-PE fluorescence (Figure S2). The specific fluorescence fractions were obtained after the subtraction of nonspecific fluorescence in the control samples (labelled with proper isotype control). Gates for PE and FITC fluorescents were estimated based on the fluorescence of unstained probes. The percentage values of CD61+/CD45+ positive objects (PLAs) were calculated relative to the total number of leucocytes (CD45 positive cells) present in each sample. All of the data analysis was performed in CyFlow version 1.5.1.2 (Pertec, Görlitz, Germany).

2.5. Cytokine Level Analysis

After preparation, the samples were centrifuged (2500× *g*, 15 min) to obtain plasma. The following cytokine levels: Interleukin 2 (IL-2), tumuor necrosis factor (TNF), interferon α (INF-α), interferon γ (INF-γ), transforming growth factor β (TGF-β), were all measured using commercial ELISA kits (Mabtech, Nacka Strand, Sweden) in accordance with the manufacturer’s protocol. All of the measurements were made using MaxiSorp plates (Nunv, Roskilde, Denmark). Absorbance was measured at 450 nm using the SPECTROstar Nano Microplate Reader (BMG Labtech, Ortenberg, Germany).

2.6. Isolation of RNA and Reverse Transcription

Frozen whole blood samples (−80 °C) were lysed using TRI Reagent® (Sigma-Aldrich), after which separation was performed. Then the InviTrap Spin Universal RNA Mini Kit (Stratec Biomedical Systems, Birkenfeld, Germany) was used to purify the RNA-containing aqueous phase. The quantity and purity of RNA were estimated using a Synergy HTX Multi-Mode Microplate Reader equipped with a Take3 Micro-Volume Plate (BioTek Instruments, Inc., Winooski, VT, USA). Total RNA (0.15 μg) was reverse transcribed into cDNA with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Waltham, MA, USA). All of the steps were performed according to the manufacturer's recommendations.

2.7. Real-Time PCR

Expression levels of both studied genes were obtained using the following TaqMan probes: Hs00174128_m1 for the human *TNF* gene, Hs00989291_m1 for the human *INF-γ* gene, and Hs99999901_s1 as an endogenous control, which was the human *18S rRNA* gene (Life Technologies, Carlsbad, CA, USA). Real-time PCR analyses were performed using a CFX96 real-time PCR system (BioRad Laboratories, Hercules, CA, USA) with a TaqMan Universal Master Mix II, without UNG (Life Technologies). All procedures were performed according to the manufacturers' protocols. Relative expressions of the studied genes were calculated using the equation $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{18S\ rRNA}$.

2.8. Statistical Analysis

All experiments were performed in duplicate, calculated as mean values and expressed as mean ± SD. All-statistical analyses were performed using Stats Direct statistical software Version. 2.7.2 (StatsDirect software, Cheshire, UK). The results obtained were analysed for normality using a Shapiro-Wilk test. Next, the results were analysed for equality of variance using Levene's test. The significance of the differences between the values was analysed using ANOVA, followed by Tukey's range test for multiple comparisons (for data with normal distribution and equality of variance), and the Kruskal-Wallis test; $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Flavonolignans Effect on IL-1β-Induced Formation of Blood Platelet-Leukocyte Aggregates

To determine the level of interaction between blood platelets and leucocytes we applied double-label flow cytometry as an investigative method. Based on flow cytometry measurements, our results clearly indicate that IL-1β is statistically significant ($p < 0.001$) in the induction (about three times–6.9% vs. 22.8%) of the formation of platelets-leukocytes aggregates (Figure 1). Next, we observed that, dose dependent, two of the three tested flavonolignans–silychristin and silybin–inhibit the IL-1β-induced formation of blood platelet-leukocyte aggregates in the whole blood samples (Figure 2). In the highest used concentration (100 μM), it was observed that both silychristin and silybin are able to reduce the formation of platelet-leukocyte aggregate formation in IL-1β-induced samples to similar values, as observed in the control samples (without IL-1β)—22.8% vs. 7.5% and 7.6%, respectively.

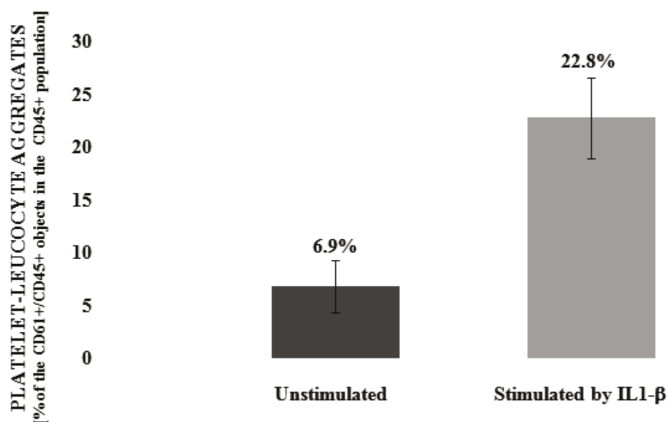


Figure 1. The effect of IL-1β (10 ng/mL) on the formation of blood platelet-leukocyte aggregates. Results of double-label flow cytometry measurements are expressed as the amount of CD61+/CD45+ objects in the whole CD45+ population (presented as %), $n = 12$, $p < 0.001$.

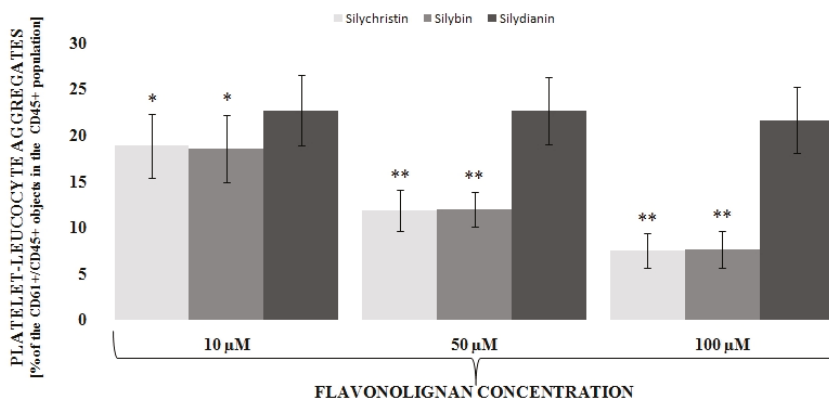


Figure 2. The effect flavonolignans; silychristin, silybin and silydianin in concentrations of 10, 50, and 100 μM on the IL-1β (10 ng/mL) induced the formation of blood platelet-leukocyte aggregates. The results of double-label flow cytometry measurements are expressed as the amount of CD61+/CD45+ objects in the whole CD45+ population (presented as %), $n = 12$; * $p < 0.01$, ** $p < 0.001$.

3.2. Flavonolignans Effect on IL-1β-Induced Cytokine Production (IL-2, TGF-β, TNF, INF-α and INF-γ)

In the next step, we determined the effects of flavonolignans on IL-1β-induced cytokine production by blood cells using the ELISA method. For this analysis, we selected 5 cytokines: IL-2, TGF-β, TNF, INF-α, and INF-γ. In all of them, except for TGF-β, after IL-1β treatments of blood samples a statistically significant increase was observed. The highest induction of production by IL-1β was observed for TNF (about 5 times—from 355 pg/mL to 1632 pg/mL), and INF-γ (about 7 times—from 255 pg/mL to 1701 pg/mL). Subsequently, we evaluated the inhibitory effect of flavonolignans on the IL-1β-induced production of cytokines by blood cells. In all samples in which the blood had been treated with silybin and silychristin, a reduction of cytokine concentration was observed (Table 1). In samples treated with 100 μM silychristin and 100 μM silybin, the levels of produced cytokines were reduced by about 90%. In samples treated with silydianin, we observed some inhibitory tendencies, however, none were statistically significant.

Table 1. The effect of flavonolignans; silychristin, silybin and silydianin in concentrations of 10, 50, and 100 μM on the IL-1β (10 ng/mL) induced the production of pro-inflammatory cytokines (IL-2, TNF, INF-α, INF-γ, TGF-β). The levels of pro-inflammatory cytokines were estimated in plasma obtained from whole blood samples treated with IL-1β and flavonolignans and presented as a mean of concentration ± SD, $n = 12$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cytokine	Control (without IL1-β)	Control (with IL1-β)	IL1-β + Silychristin (μM)			IL1-β + Silybin (μM)			IL1-β + Silydianin (μM)		
			10	50	100	10	50	100	10	50	100
INF-γ (pg/mL)	255 ± 90	1701 ± 411 ***	1278 ± 339 **	555 ± 175 ***	262 ± 82 ***	1339 ± 337 **	574 ± 149 ***	283 ± 87 ***	1677 ± 398	1665 ± 483	1542 ± 405
TNF (pg/mL)	355 ± 110	1632 ± 473 ***	1216 ± 396 **	478 ± 160 ***	338 ± 119 ***	1286 ± 408 *	466 ± 115 ***	352 ± 115 ***	1614 ± 453	1610 ± 423	1494 ± 360
INF-α (pg/mL)	13.3 ± 6.4	20.8 ± 8.0 **	16.8 ± 6.1	15.0 ± 4.9 *	13.3 ± 4.0 ***	16.3 ± 4.4 *	14.8 ± 4.5 *	12.6 ± 3.5 ***	20.1 ± 5.1	18.8 ± 4.4	17.9 ± 3.9
IL-2 (pg/mL)	116 ± 28	189 ± 49 **	168 ± 45	129 ± 19 **	116 ± 24 ***	164 ± 38	135 ± 21*	112 ± 17 ***	191 ± 46	175 ± 31	172 ± 30
TGF-β (pM)	78.3 ± 23.3	86.8 ± 27.9	84.2 ± 24.1	83.6 ± 28.1	83.6 ± 27.9	86.8 ± 31.8	82.8 ± 19.2	82.3 ± 27.7	84.1 ± 24.5	87.2 ± 23.5	85.3 ± 25.1

3.3. Silychristin and Silybin Effect on mRNA Expression for INF-γ and TNF Genes

In order to evaluate the mechanism of silychristin and silybin anti-inflammatory effects, we performed a gene expression analysis at the mRNA level using the Real-Time PCR method. We decided to evaluate changes in mRNA expression for INF-γ and TNF genes, for which expression was induced by IL-1β (about 25 [0.0101 vs. 0.2473] and 11 [0.0462 vs. 0.508] times, respectively). In our measurements, we found that, in a dose-dependent manner, both silychristin and silybin inhibit the IL-1β-induced expression of INF-γ and TNF genes at the mRNA level (Figures 3 and 4). Both compounds have similar inhibitory effects, with the strongest observed at the highest concentration (100 μM), in which gene expressions were reduced to a level observed in the control samples without IL-1β treatment.

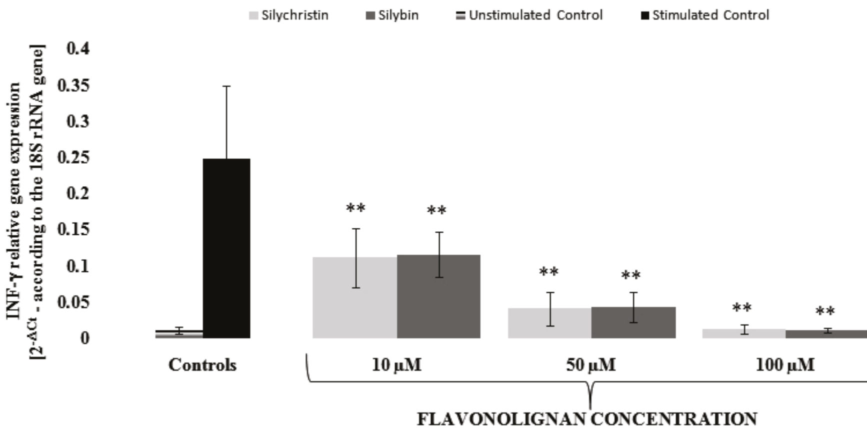


Figure 3. The effect of flavonolignans; silychristin and silybin in concentrations of 10, 50, and 100 μM on the IL-1β (10 ng/mL) induced the expression of *INF-γ* gene (measured at the mRNA level). The results are expressed as a mean of $2^{-\Delta ct}$ (according to the reference gene-*18S rRNA*) ± SD, *n* = 12; ** *p* < 0.001.

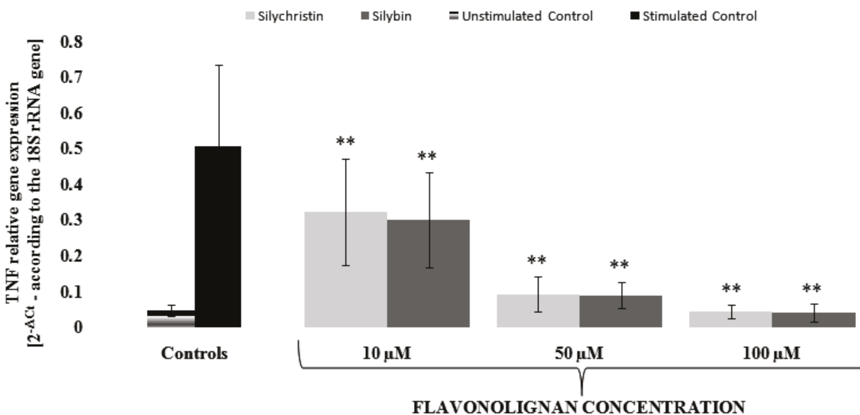


Figure 4. The effect of flavonolignans; silychristin and silybin in concentrations of 10, 50, and 100 μM on the IL-1β (10 ng/mL) induced the expression of *TNF* gene (measured at the mRNA level). The results are expressed as a mean of $2^{-\Delta ct}$ (according to the reference gene—18*S rRNA*) ± SD, *n* = 12; ** *p* < 0.001.

4. Discussion

Thrombosis and inflammation are closely related pathophysiological processes with multicellular activation involving blood platelets and leukocytes. Coronary artery disease, including Acute Coronary Syndromes (ACS), which refers to group of clinical symptoms is compatible with acute myocardial ischaemia, associated with coronary artery thrombosis is one of the most common causes of death in the world. It is now believed that elevated levels of inflammatory factors in the blood promote the development of cardiovascular events, and that chronic inflammation plays a key role in the pathogenesis of atherosclerosis and acute coronary syndromes. Much intensive study by various leading scientific centres around the world confirms that the balance between pro-and anti-inflammatory processes influence the risk of developing ACS [21]. In blood samples obtained from persons with acute coronary syndrome episodes, elevated concentrations of chemokines (IL-8, MCP-1, eotaxin, MIP-1α, and IP-10) and cytokines (IL-1, IL-6, IL-7, IL-12, IL-17, IFN-α, and granulocyte-macrophage colony-stimulating factor) regulating both innate and adaptive immunity have been observed [22].

Numerous studies form cardiovascular disease have shown that the platelet-leukocyte interaction (so-called crosstalk) was increased [23]. Thrombosis and inflammation involve complex platelet-leukocyte interaction, the details of which have not been fully elucidated. Under conditions that mimic a physiological state, the platelet-leukocyte cross-talk involves multiple mediators and mechanisms [24], and is a common feature of atherothrombosis and inflammatory immune reactions. In the last few years, it has been suggested that platelet-leukocyte interactions contribute to cardiovascular disease [25]. The creation of PLAs involves the recruitment leukocytes to the atherosclerotic plaques and stimulates them to release collagenases such as MMP-8, MMP-9, and proteinase 2, which affect the reduction of the atherosclerotic plaque stability by degrading the collagen of the extracellular matrix [26]. As a result of pathological platelet activation, there is an increased immune response and an increasing number of platelet-leukocyte complexes formed at the site of the atherosclerotic plaque, which can lead to its rupture [27]. An increased number of platelet-leukocyte aggregates circulating in blood have been observed in patients with ACS [28]. Additionally, in patients who died following an ACS episode, platelet-neutrophil interactions occurring at the site of ruptured plaques have been observed [29]. Flow-Cytometric analysis of platelet aggregation showed the significant effect of IL-1β on the formation of PLAs, showed the significant effect of IL-1β on the formation of PLAs, which suggests that IL-1β significantly affects pro-inflammatory and prothrombotic cross-talk between platelets and leukocytes. In the current study, we have also observed the very strong ability of IL-1β to induce the formation of blood platelet-leukocyte aggregates (Figure 1). However, this effect was abolished with the application of two flavonolignans: silychristin and silybin. Depending on the dose, these two inhibit the formation of blood platelet-leukocyte aggregates induced by IL-1β (Figure 2).

Leukocytes enhance the inflammatory process within the atherosclerotic plaque's formation. Pro-inflammatory cytokines, such as TNF and INF-γ, as inducers of endothelial cell activation and expression of adhesive particles, play a key role in the recruitment of leukocytes, particularly monocytes, from the blood stream [30]. TNF and IFN-γ are particularly toxic to endothelial cells, and activate monocytes, macrophages, and phospholipase A2, which intensifies the synthesis of pro-inflammatory eicosanoids in the atherosclerotic plaque [31]. Additionally, these inflammatory mediators exacerbate the expression of MMPs in macrophages, as well as in endothelial and smooth muscle cells. MMP activity, regulated by inflammatory mediators, is responsible for the disintegration of interstitial collagen, leading to thinning and fibrous cup (FC) depletion, causing a susceptibility to atherosclerotic plaque's fracture [32]. INF-γ also inhibits the ability of smooth muscle cells to synthesize the new interstitial collagen fibres required for the repair of the FC extracellular matrix [33].

In our study, we observed that of all the tested cytokines (IL-2, TNF, INF-α, INF-γ, TGF-β), IL-1β most induces the production of TNF and INF-γ (Table 1). Additionally, in this study, we have observed that the two tested flavonolignans: silybin and silychristin, reduce the IL-1β-induced

production of cytokines. In the highest concentration of the tested compounds (100 μM), we observed the almost complete abolition of the pro-inflammatory action of IL-1β.

Contrary to numerous in vitro and in vivo studies on flavonolignans, including investigations of hepatoprotective activity [34], their anti-inflammatory properties and the therapeutic effects have been less thoroughly described. However, some information is available [35]. The biochemical mechanisms include the modulation of a variety of cell-signalling pathways, resulting in the reduction of pro-inflammatory mediators. Both silymarin [36] and pure silybin [37] suppress NF-κB, which plays a crucial role in regulating immune response and inflammation through the regulation of the expression of various genes involved in these processes [38]. A non-activated NF-κB is maintained in the cytoplasm by the inhibitory protein 1-κBα (IκBα). The activation of NF-κB occurs via the phosphorylation of IκBα, leading to its proteasome-mediated degradation, release from IκBα complex, and the translocation to the nucleus. NF-κB pathway plays an essential role in activating genes encoding pro-inflammatory cytokines (TNF, IFN, IL-1β, IL-2, IL-6, and granulocyte macrophage colony-stimulating factors), chemokines (IL-8, macrophage inflammatory protein 1α, macrophage chemotactic protein 1), enzymes that generate mediators of inflammation (5-lipoxygenase), immune receptors (interleukin-2 receptors), and also adhesion molecules (E-selectin, intercellular adhesion molecule 1) [39]. The study presented by Trappoliete et al. [40], shows that silybin is able to inhibit the IκBα phosphorylation, which suppresses the IL-1β-induced activation of the NF-κB pathway in hepatic stellate cells (HSC). Silybin was also able to suppress the antigen-stimulated calcium uptake and the activation of NF-κB, resulting in the significant reduction of TNF and IL-6 production [41]. In the present study, in samples treated with silybin and silychristin, we observed a reduction of the levels of cytokines secreted into plasma, as well as the inhibition of IL-1β-induced expression of TNF and IFN-γ, which confirms the anti-inflammatory effect of these two flavonolignans. Additionally, in the last few years, novel forms of flavonolignans administration have been developed that posses a very high bioavailability (with plasma concentrations ranging from 60–70 μM) [42]. This corresponds with the concentrations of flavonolignans that have a biological effect in our study (10–100 μM).

5. Conclusions

In summary, our results indicate that flavonolignans may be used in the prevention of cardiovascular disease with dual action as antiplatelet and anti-inflammatory agents. Further studies using a larger sample size and additional studies, demonstrating NF-κB activity is necessary before the final statement about the role of the inhibitory effect of these two flavonolignans on the NF-κB pathway.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/9/1022/s1. Figure S1: Chemical structures of flavonolignans used in this study, Figure S2: Flow cytometry dot plot presented blood platelet-leukocyte aggregates.

Acknowledgments: This study was supported by grants: B1611000001144.02, B161100000004601 and grant 506/1136 from the University of Lodz.

Author Contributions: M.B. (corresponding author) and J.S.-B. conceived and designed the study; M.B. prepared all samples and collected and analyzed the data; M.B., A.D., E.S. performed research analysis; All co-authors wrote the paper, have read and approved the final manuscript

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Dinarello, C.A. Biologic basis for interleukin-1 in disease. *Blood* **1996**, *87*, 2095–2147. [PubMed]
2. Weber, A.; Wasiliew, P.; Kracht, M. Interleukin-1beta (IL-1beta) processing pathway. *Sci. Signal.* **2010**, *3*, cm2. [CrossRef] [PubMed]
3. Kleiner, G.; Marcuzzi, A.; Zanin, V.; Monasta, L.; Zauli, G. Cytokine levels in the serum of healthy subjects. *Mediat. Inflamm.* **2013**, *2013*, 434010. [CrossRef] [PubMed]

4. Weber, A.; Wasiliew, P.; Kracht, M. Interleukin-1 (IL-1) pathway. *Sci. Signal.* **2010**, *3*, cm1. [CrossRef] [PubMed]
5. Beaulieu, L.M.; Lin, E.; Mick, E.; Koupenova, M.; Weinberg, E.O.; Kramer, C.D.; Genco, C.A.; Tanriverdi, K.; Larson, M.G.; Benjamin, E.J.; et al. Interleukin 1 receptor 1 and interleukin 1beta regulate megakaryocyte maturation, platelet activation, and transcript profile during inflammation in mice and humans. *Arterioscler. Thromb. Vasc. Biol.* **2014**, *34*, 552–564. [CrossRef] [PubMed]
6. Dinarello, C.A. Blocking IL-1 in systemic inflammation. *J. Exp. Med.* **2005**, *201*, 1355–1359. [CrossRef] [PubMed]
7. Abenavoli, L.; Capasso, R.; Milic, N.; Capasso, F. Milk thistle in liver diseases: Past, present, future. *Phytother. Res.* **2010**, *24*, 1423–1432. [CrossRef] [PubMed]
8. Rainone, F. Milk thistle. *Am. Fam. Phys.* **2005**, *72*, 1285–1288.
9. Althagafy, H.S.; Meza-Avina, M.E.; Oberlies, N.H.; Croatt, M.P. Mechanistic study of the biomimetic synthesis of flavonolignan diastereoisomers in milk thistle. *J. Org. Chem.* **2013**, *78*, 7594–7600. [CrossRef] [PubMed]
10. Kurkin, V.A. Phenylpropanoids from medicinal plants: Distribution, classification, structural analysis, and biological activity. *Chem. Nat. Comp.* **2003**, *39*, 123–153. [CrossRef]
11. Nyiredy, S.; Samu, Z.; Szucs, Z.; Gulacsi, K.; Kurtan, T.; Antus, S. New insight into the biosynthesis of flavanolignans in the white-flowered variant of Silybum marianum. *J. Chromatogr. Sci.* **2008**, *46*, 93–96. [CrossRef] [PubMed]
12. Kren, V.; Walterova, D. Silybin and silymarin–new effects and applications. *Biomed. Pap. Med. Fac. Univ. Palacky. Olomouc. Czech. Repub.* **2005**, *149*, 29–41. [CrossRef] [PubMed]
13. Gazak, R.; Walterova, D.; Kren, V. Silybin and silymarin—New and emerging applications in medicine. *Curr. Med. Chem.* **2007**, *14*, 315–338. [CrossRef] [PubMed]
14. Kim, N.C.; Graf, T.N.; Sparacino, C.M.; Wani, M.C.; Wall, M.E. Complete isolation and characterization of silybins and isosilybins from milk thistle (*Silybum marianum*). *Org. Biomol. Chem.* **2003**, *1*, 1684–1689. [CrossRef] [PubMed]
15. Hackett, E.S.; Twedt, D.C.; Gustafson, D.L. Milk thistle and its derivative compounds: A review of opportunities for treatment of liver disease. *J. Vet. Intern. Med.* **2013**, *27*, 10–16. [CrossRef] [PubMed]
16. Lee, J.I.; Narayan, M.; Barrett, J.S. Analysis and comparison of active constituents in commercial standardized silymarin extracts by liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B* **2007**, *845*, 95–103. [CrossRef] [PubMed]
17. Bijak, M. Flavonolignans-compounds not only for liver treatment. *Pol. Merkur. Lek.* **2017**, *42*, 34–37.
18. Bijak, M.; Szelenberger, R.; Saluk, J.; Nowak, P. Flavonolignans inhibit ADP induced blood platelets activation and aggregation in whole blood. *Int. J. Biol. Macromol.* **2017**, *95*, 682–688. [CrossRef] [PubMed]
19. Bijak, M.; Saluk-Bijak, J. Flavonolignans inhibit the arachidonic acid pathway in blood platelets. *BMC Complement. Altern. Med.* **2017**, *17*, 396. [CrossRef] [PubMed]
20. Bijak, M.; Dziedzic, A.; Saluk-Bijak, J. Flavonolignans reduce the response of blood platelet to collagen. *Int. J. Biol. Macromol.* **2017**. [CrossRef] [PubMed]
21. Lach, D.; Cichon, N.; Dziedzic, A.; Bijak, M.; Saluk, J. Inflammatory processes in the pathogenesis of acute coronary syndromes. *Pol. Merkur. Lek.* **2017**, *42*, 183–186.
22. Wyss, C.A.; Neidhart, M.; Altwegg, L.; Spanaus, K.S.; Yonekawa, K.; Wischnewsky, M.B.; Corti, R.; Kucher, N.; Roffi, M.; Eberli, F.R.; et al. Cellular actors, Toll-like receptors, and local cytokine profile in acute coronary syndromes. *Eur. Heart J.* **2010**, *31*, 1457–1469. [CrossRef] [PubMed]
23. Zeller, J.A.; Lenz, A.; Eschenfelder, C.C.; Zunker, P.; Deuschl, G. Platelet-leukocyte interaction and platelet activation in acute stroke with and without preceding infection. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 1519–1523. [CrossRef] [PubMed]
24. Li, N.; Hu, H.; Lindqvist, M.; Wikstrom-Jonsson, E.; Goodall, A.H.; Hjemdahl, P. Platelet-leukocyte cross talk in whole blood. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 2702–2708. [CrossRef] [PubMed]
25. Totani, L.; Evangelista, V. Platelet-leukocyte interactions in cardiovascular disease and beyond. *Arterioscler. Thromb. Vasc. Biol.* **2010**, *30*, 2357–2361. [CrossRef] [PubMed]
26. Kral, J.B.; Schrottmaier, W.C.; Salzmann, M.; Assinger, A. Platelet Interaction with Innate Immune Cells. *Transfus. Med. Hemother.* **2016**, *43*, 78–88. [CrossRef] [PubMed]

27. Furman, M.I.; Barnard, M.R.; Krueger, L.A.; Fox, M.L.; Shilale, E.A.; Lessard, D.M.; Marchese, P.; Frelinger, A.L.; Goldberg, R.J.; Michelson, A.D. Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. *J. Am. Coll. Cardiol.* **2001**, *38*, 1002–1006. [CrossRef]
28. Sarma, J.; Laan, C.A.; Alam, S.; Jha, A.; Fox, K.A.; Dransfield, I. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* **2002**, *105*, 2166–2171. [CrossRef] [PubMed]
29. Naruko, T.; Ueda, M.; Haze, K.; van der Wal, A.C.; van der Loos, C.M.; Itoh, A.; Komatsu, R.; Ikura, Y.; Ogami, M.; Shimada, Y.; et al. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation* **2002**, *106*, 2894–2900. [CrossRef] [PubMed]
30. Libby, P.; Lichtman, A.H.; Hansson, G.K. Immune effector mechanisms implicated in atherosclerosis: From mice to humans. *Immunity* **2013**, *38*, 1092–1104. [CrossRef] [PubMed]
31. Nakajima, T.; Schulte, S.; Warrington, K.J.; Kopecky, S.L.; Frye, R.L.; Goronzy, J.J.; Weyand, C.M. T-cell-mediated lysis of endothelial cells in acute coronary syndromes. *Circulation* **2002**, *105*, 570–575. [CrossRef] [PubMed]
32. Libby, P. Collagenases and cracks in the plaque. *J. Clin. Investig.* **2013**, *123*, 3201–3203. [CrossRef] [PubMed]
33. Amento, E.P.; Ehsani, N.; Palmer, H.; Libby, P. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler. Thromb.* **1991**, *11*, 1223–1230. [CrossRef] [PubMed]
34. Loguercio, C.; Festi, D. Silybin and the liver: From basic research to clinical practice. *World J. Gastroenterol.* **2011**, *17*, 2288–2301. [CrossRef] [PubMed]
35. Hussain, S.A.; Jassim, N.A.; Numan, I.T.; Al-Khalifa, I.I.; Abdullah, T.A. Anti-inflammatory activity of silymarin in patients with knee osteoarthritis. A comparative study with piroxicam and meloxicam. *Saudi Med. J.* **2009**, *30*, 98–103. [PubMed]
36. Manna, S.K.; Mukhopadhyay, A.; Van, N.T.; Aggarwal, B.B. Silymarin suppresses TNF-induced activation of NF-kappa B, c-Jun N-terminal kinase, and apoptosis. *J. Immunol.* **1999**, *163*, 6800–6809. [PubMed]
37. Bannwart, C.F.; Nakaira-Takahagi, E.; Golim, M.A.; de Medeiros, L.T.; Romao, M.; Weel, I.C.; Peracoli, M.T. Downregulation of nuclear factor-kappa B (NF-kappaB) pathway by silibinin in human monocytes challenged with Paracoccidioides brasiliensis. *Life Sci.* **2010**, *86*, 880–886. [CrossRef] [PubMed]
38. Ramasamy, K.; Agarwal, R. Multitargeted therapy of cancer by silymarin. *Cancer Lett.* **2008**, *269*, 352–362. [CrossRef] [PubMed]
39. Lawrence, T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a001651. [CrossRef] [PubMed]
40. Trappoliere, M.; Caligiuri, A.; Schmid, M.; Bertolani, C.; Failli, P.; Vizzutti, F.; Novo, E.; di Manzano, C.; Marra, F.; Loguercio, C.; et al. Silybin, a component of sylimarin, exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells. *J. Hepatol.* **2009**, *50*, 1102–1111. [CrossRef] [PubMed]
41. Choi, Y.H.; Yan, G.H. Silibinin attenuates mast cell-mediated anaphylaxis-like reactions. *Biol. Pharm. Bull.* **2009**, *32*, 868–875. [CrossRef] [PubMed]
42. Parveen, R.; Baboota, S.; Ali, J.; Ahuja, A.; Vasudev, S.S.; Ahmad, S. Oil based nanocarrier for improved oral delivery of silymarin: In vitro and in vivo studies. *Int. J. Pharm.* **2011**, *413*, 245–253. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Polyphenols from Root, Tubercles and Grains Cropped in Brazil: Chemical and Nutritional Characterization and Their Effects on Human Health and Diseases

Diego dos Santos Baião, Cyntia Silva de Freitas, Laidson Paes Gomes, Davi da Silva, Anna Carolina N. T. F. Correa, Patricia Ribeiro Pereira, Eduardo Mere Del Aguila and Vania Margaret Flosi Paschoalin *

Instituto de Química, Universidade Federal do Rio de Janeiro, Cidade Universitária Av Athos da Silveira Ramos 149, 21949-909 Rio de Janeiro (RJ), Brazil; diegobaiao20@hotmail.com (D.d.S.B.); cyntia.freitas@yahoo.com.br (C.S.d.F.); laidsonpaes@gmail.com (L.P.G.); daviuerj@gmail.com (D.d.S.); annac.correa@hotmail.com (A.C.N.T.F.C.); biopatbr@gmail.com (P.R.P.); emda@iq.ufrj.br (E.M.D.A.)

* Correspondence: paschv@iq.ufrj.br; Tel.: +55-21-3938-7362; Fax: +55-21-3938-7266

Received: 3 August 2017; Accepted: 13 September 2017; Published: 20 September 2017

Abstract: Throughout evolution, plants have developed the ability to produce secondary phenolic metabolites, which are important for their interactions with the environment, reproductive strategies and defense mechanisms. These (poly)phenolic compounds are a heterogeneous group of natural antioxidants found in vegetables, cereals and leguminous that exert beneficial and protective actions on human health, playing roles such as enzymatic reaction inhibitors and cofactors, toxic chemicals scavengers and biochemical reaction substrates, increasing the absorption of essential nutrients and selectively inhibiting deleterious intestinal bacteria. Polyphenols present in some commodity grains, such as soy and cocoa beans, as well as in other vegetables considered security foods for developing countries, including cassava, taro and beetroot, all of them cropped in Brazil, have been identified and quantified in order to point out their bioavailability and the adequate dietary intake to promote health. The effects of the flavonoid and non-flavonoid compounds present in these vegetables, their metabolism and their effects on preventing chronic and degenerative disorders like cancers, diabetes, osteoporosis, cardiovascular and neurological diseases are herein discussed based on recent epidemiological studies.

Keywords: beetroot; cassava; taro; cocoa nibs; soybeans and antioxidant activity

1. Introduction

Natural polyphenols are secondary metabolites found in vegetables and edible plants, cereals, fruit, seeds, oils and products manufactured from foods such as non-alcoholic and alcoholic beverages, like tea and red wine, and, mainly, cocoa products [1,2]. In plants, polyphenols are involved in the protective response to different stresses, including abiotic stresses, such as ultraviolet radiation where these compounds can impair oxidative stress and DNA damage, in injuries to the vegetal body where polyphenols stimulate the lignification process, contributing to healing, or in the defense against aggression by pathogens, where their concentrations may be increased after infection by virus, fungus, bacteria and even nematodes [3]. In foods, these compounds may contribute to bitterness, astringency, color, flavor, odor and oxidative stability [4].

Systematic reviews and meta-analysis studies have shown that dietary polyphenol intake may be associated with a decreased risk of chronic and degenerative diseases.

After evaluating 22 prospective studies, Grosso et al., 2017 [5] concluded that the high consumption of total flavonoids decreased the risk of all-cause mortality. However, a 100-mg/day increment in flavonoid intake led to near to 5% decreased risk of all-cause and CVD (cardiovascular disease) mortality. Data from 18 human randomized controlled trials regarding flavonol supplementation were pooled and some heterogeneity was observed in the individual physiological responses of cardiometabolic biomarkers, such as blood lipids, blood pressure and plasma glucose [6]. Variability in the response of blood lipids to supplementation with flavonols was found, although significant reductions in total cholesterol, LDL (low-density lipoprotein) cholesterol and triacylglycerol, as well as a significant increase in HDL (high-density lipoprotein) cholesterol was a general effect among individuals. A significant reduction was also observed in fasting plasma glucose and in systolic and diastolic blood pressures. The reduction in CVD risk after flavonol supplementation was more pronounced in participants with diagnosed disease or dyslipidemia [6].

Cardioprotective effects were obtained for intakes of six classes of flavonoids, namely flavonols, anthocyanidins, proanthocyanidins, flavones, flavanones and flavan-3-ols [5–7]. Polyphenol dietary intake may be also associated with reduced risk for type 2 diabetes. A meta-analysis of 6 prospective cohorts involved 18,146 cases and 284,800 participants found that consumption of dietary total flavonoids is associated with a reduced risk of type 2 diabetes. Beneficial effects of polyphenol consumption were observed in the US population in men aged >40 years old people and in studies ≥20 years in duration [8]. Certainly, the reduction on diabetes risk contributes to the overall risk reduction of CVD.

It is estimated that at least 8000 polyphenols have been already described, considering natural, semi-synthetic or synthetic compounds, and this broad set of structures underlies the unpaired physico-chemical and biological properties displayed by each class of compounds. Polyphenols can be classified into main classes including phenolic acids, flavonoids, stilbenes and lignans. However, food matrices generally contain a complex mixture of those compounds, at variable concentrations, which may not be well characterized.

To review existing knowledge on selected polyphenol-rick foods characteristic of the Brazilian region, the bioactivity of polyphenols present in root and tubercle vegetables, such as taro, beetroot and cassava, and in grains, like soybean and cocoa, will be discussed. Some of these vegetables and grains are highly consumed commodities and considered valuable crops worldwide. The effects of these polyphenols will be explored, such as their role in preventing degenerative disorders like cancers, diabetes, osteoporosis, and cardiovascular and neurological diseases [9].

2. Polyphenol Structural Characterization and Classification

Phenolic compounds are named when at least one hydroxyl group (–OH) is bound to one or more benzene aromatic rings (C_6H_5 or C6), forming the phenol structure (C_6H_5OH). If they exhibit a single aromatic ring, they are termed monophenolics or simple phenolics. As suggested by their nomenclature, polyphenols refer to molecules with two or more aromatic rings and at least one hydroxyl group attached to these rings, giving rise to a heterogeneous group of chemical compounds [10,11]. Based on the number of phenolic hydroxyl groups, the presence of specific functional groups attached to the benzene ring and the type of connection between rings, phenolic compounds can be divided into several classes [10] (Figure 1).

Since 1957, many attempts have been made to determine an adequate definition for the term polyphenol, however, none fits all the existing variety of polyphenols. In spite of this, a standard definition, which is a combination of the proposed definitions throughout the years, has been established, although still excluding simple phenolic compounds [11]. Based on this, the majority of scientific papers consider all phenolic compounds as a polyphenol, irrespective of the number of aromatic rings [10,12] based on the fact that they share similar properties and characteristics with those composed of multiple aromatic rings [13]. This tendency is also widespread in the agricultural, food and pharmaceutical industries, where the term polyphenols is preferred for commercial purposes, and

includes any type of phenolic compounds [11]. The present review will not differ in this point, since it will discuss polyphenols as a unique group, including the simplest ones.

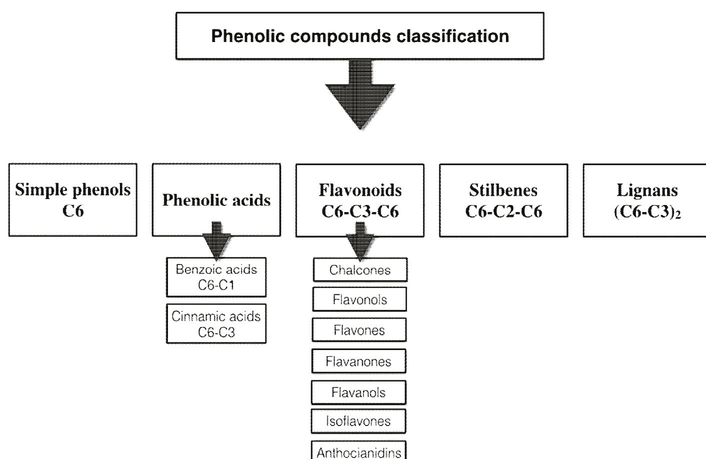


Figure 1. Schematic representation of the main classes and subclasses of phenolic compounds and their respective chemical structure backbone, where C6 corresponds to the aromatic ring and C1, C2 or C3 refers to side chains or intermediate chains.

The simplest phenolic compounds exhibit one phenol structure and up to three functional groups (R, R1 and R2) attached to the aromatic ring, but with the hydroxyl group as the main functional group. Argan (*Argania spinosa*) oil is a rich source of simple phenolic compounds, which are mainly represented by resorcinol, composed of two hydroxyl groups linked to the benzene ring. Phloroglucinol, containing an additional hydroxyl group, is another example of a simple phenolic compound, which has been extracted from *Eucalipitus kino*, *Acacia arabica* and marine algae (Figure 2) [10,14].

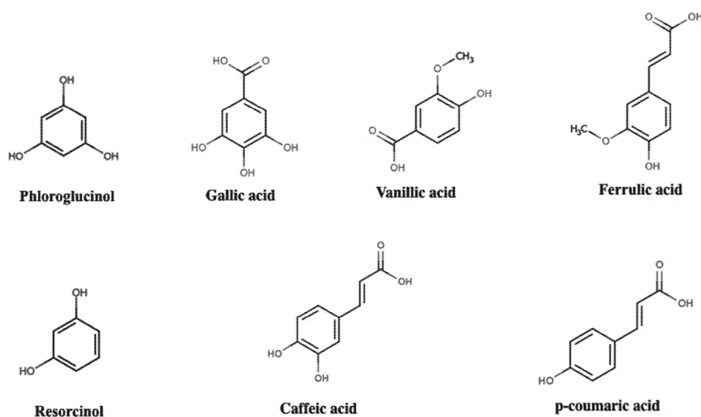


Figure 2. Chemical structures of phenolic acids, adapted from the database "Polyphenol content in foods" (http://phenol-explorer.eu). Gallic acid and vanillin acid belongs to benzoic acids subclass, characterized by a backbone composed of 7 carbons (C6-C1). Caffeic acid, ferric acid and p-coumaric acid represent the cinnamic acids subclass and exhibit a backbone composed of 9 carbons (C6-C3) [1].

The phenolic acid class can also display the same short and simple structures, but with the additional presence of a carboxyl group (–COOH) attached to the aromatic ring, besides hydroxyl or methoxyl groups, which total up to three functional groups, similarly to simple phenols. This class is divided into two subclasses, benzoic acids and cinnamic acids, characterized by a backbone containing 7 (C6-C1) and 9 (C6-C3) carbon atoms, respectively [12,15]. Benzoic acids can be represented by gallic acid (R=R1=R2=OH) and vanillic acid (R=OH, R1=OCH_3, R2=H) (Figure 2) [16].

The largest and most studied class of polyphenols is represented by flavonoids, which account for over 50% of phenolic compounds. They are composed of 15 carbon atoms that form two aromatic rings (A and B), connected by three carbons, giving rise to the typical C6-C3-C6 backbone (Figure 3). The three intermediate carbon atoms can assume different configurations, ranging from open to heterocyclic chains, condensed with the aromatic ring A. In this case, the intermediate chain is called ring C [10,15]. When ring C is arranged in the heterocyclic format, the basic phenylbenzopyran or chromane structure is generated, where benzopyran is represented by ring A (C6) fused to ring C (C3) and phenyl, by ring B (C6) [17]. Different configurations of the intermediate C3 chain and ring B will determine flavonoid classifications into their main subclasses, namely (i) chalcones; (ii) flavonols; (iii) flavones; (iv) isoflavones; (v) flavanones; (vi) anthocianidins and (vii) flavanols [12]. Several other types of substitutions (oxygenation, alkylation, glycosylation, alkylation and sulfation) in the flavonoid backbone are possible and are responsible for the high variety of more complex chemical compounds within this class [10,15]. Some flavonoids are restricted to only a few foodstuffs, such as isoflavones present in soy (about 1 mg of genistein and daidzein per g), flavonones present in citrus fruits (about 100–250 mg of hesperidin per L), flavanols present in some teas (about 200–300 mg of catechin, gallocatechin and their galloylated derivatives per g) and anthocyanins present in strawberries (4.5 mg per g of fresh fruit), beetroots (up to 19 μM trolox per mg) and red wine (26 mg per L) [18,19].

Chalcones are easily recognized among the flavonoid subclasses, since both aromatic rings are held together by an open C3 chain, while all the other six subclasses differ from each other according to the position where the ring B connects to the benzopyran portion. In most of these compounds, ring B is connected to the C2 of the heterocyclic ring C, but attachments to positions 3 and 4 can also occur (Figure 3). In some cases, chalcones are responsible for the yellow color in flowers, and are also present in fruits, such as apples, for example [12,15]. Except for the isoflavone subclass, where ring B connects to the carbon 3 of the benzopyran portion, the other compounds have their phenol portion attached to carbon 2. Isoflavones, heat sensitive compounds, are structurally similar to estrogen, being able to act as an estrogen mimetic. The richest sources of isoflavones are leguminous items, especially soybean (*Glycine max*) [20]. Flavones and flavonols exhibit a double bond linking carbons 2 and 3 of ring C, while flavonols display an additional hydroxyl group at position 3. Flavonol synthesis in plants is positively influenced by sunlight, and these compounds are easily found in the outer and aerial parts, while flavones accumulate in fruit skin but are not dependent on sunlight. Carbons 2 and 3 in flavanones and flavanols are not linked by unsaturation, but an oxygen atom is present at position 4 in flavanones and a hydroxyl group at position 3 in flavanols that differ from one another (Figure 3). Flavanones are typical constituents of citrus fruits, while flavanols are also known by their representative compound catechin, present mainly in green tea and chocolates [21,22].

Anthocyanidins differ from other polyphenols by positively charged oxygen, also called a pyrylium cation, in ring C (Figure 3). These molecules are usually associated to glycans and, in this case, are called anthocyanins. They are responsible for producing color in some plant parts, such as flowers, leaves and fruits, ranging from red to purple. Color production is dependent on pH, the presence of metal ions, type of attached sugar or acylester and anthocyanidin mixture. Two variations are possible in anthocyanidin chemical structure, including the presence of methyl groups at carbon 5 or 7 and the absence of a hydroxyl group in carbon 3 [21]. Rich sources of anthocyanidins include colorful fruits and vegetables, such as berries, grapes and red/purple vegetables [23].

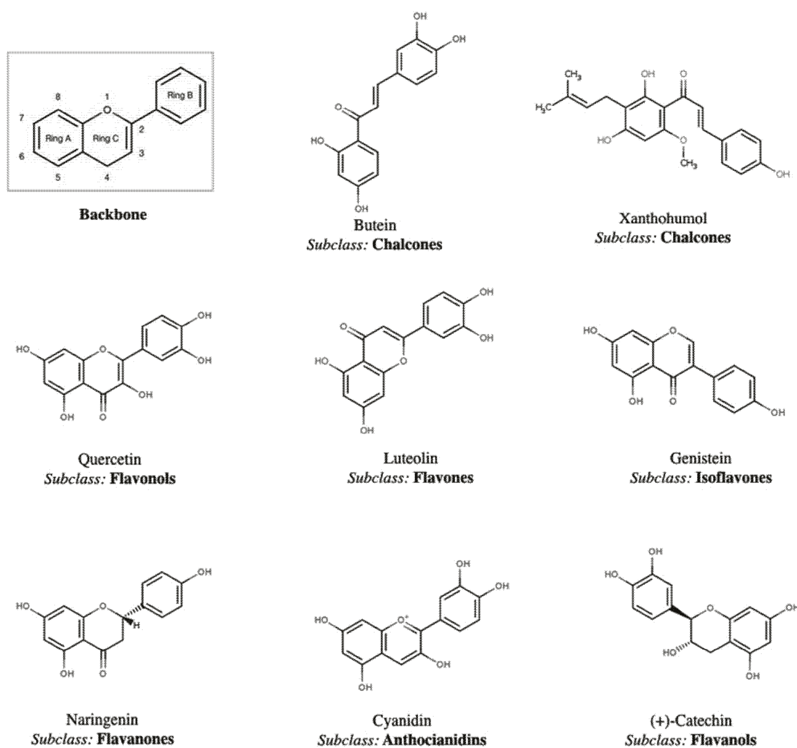


Figure 3. Representative chemical structures of the main subclasses and basic structure of the majority of flavonoids, adapted from the database "Polyphenol content in foods" (http://phenol-explorer.eu) [1].

Minor classes are represented by stilbenes and lignans, which, despite their importance to agro-food and pharmaceutical industries, are less widespread and found in few food products, mainly wines, some berry fruits and their juices. This class has a backbone quite similar to flavonoids, with the difference that both rings (A and B) are connected by a chain of two carbons (C6-C2-C6), which can occur in monomeric or oligomeric forms, and are represented by the widely known resveratrol, a typical wine constituent (Figure 4). The compounds generally derived from resveratrol belonging to class occur in their free form or associated with glycans [10,22]. Lignans originate from the dimerization of derivatives from C6-C3 compounds, such as p-coumaryl, conferyl and sinapyl alcohol, originating the typical lignan $(C6\text{-}C3)_2$ subunit backbone [17]. In classical lignans, the dimer is stabilized by the interaction between at least two carbons 8, also named Cβ by some authors, from the side chain C3. Subunit connections involving carbons other than Cβ originate non-classical lignans, or neolignans. Oxygenation of at least one of the aromatic rings is also observed in this class [24]. Lignans are found in dietary fibers, especially flax seed and tea, which make them important compounds in the prevention of some types of cancer. This class is also known as phytoestrogens, since these compounds are converted into enterolactone and enterodiol after ingestion by humans, and then act in controlling sex hormone levels by inhibiting estrogen synthesis, which is directly related to the development and progression of cancer in endocrine-related reproductive tissues [25].

Many other phenolic compounds can also be found in plant-derived food, albeit in small amounts, and, for this reason are mostly not included in the general phenolic compoundclassification. However, some authors also include coumarins [17] and tannins into this classification. Tannins were the first phenol compounds to be reported and extensively studied by scientists. These molecules are ellagic or

gallic acid polymers, or both, with a glucose molecule attached, and can also be found as a condensed form of two flavan-3-ol molecules. Considered anti-nutritional compounds, tannins have the ability to bind proteins of animal origin preventing putrefaction, which makes them powerful tools to convert animal skin into leather [17,22]. Coumarins, like cinnamic acids (Figure 2), exhibit the C6-C3 backbone, but the C3 chain is arranged as a heterocycle, formed by the presence of an oxygen atom, and can be represented by warfarin, a powerful anticoagulant compound [10].

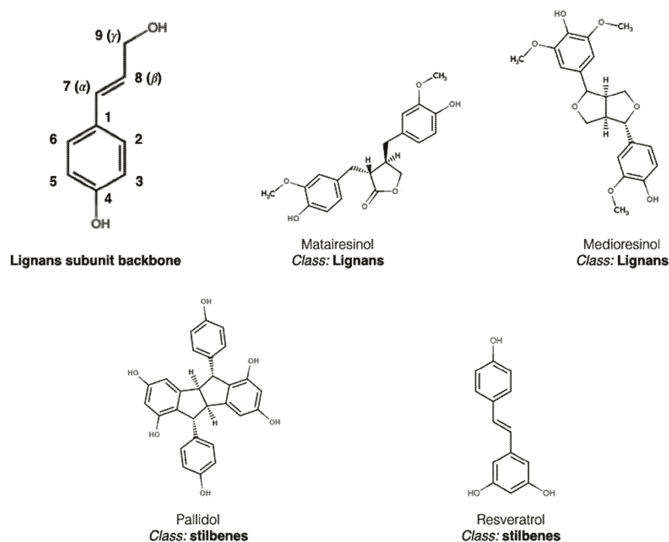


Figure 4. Chemical structures of the stilbene and lignan classes, adapted from the database "Polyphenol content in foods" (http://phenol-explorer.eu) [1].

In the last years, a significant number of studies have suggested that the extended consumption of vegetables or foods rich in polyphenols fulfill physiological needs and confer significant benefits to human health, such as combating oxidative stress, working as adjuvants in reducing the risk of developing chronic diseases, like cardiovascular conditions and protecting against the development of cancers, infections, aging, asthma and neurodegenerative diseases [26,27].

A significant number of studies, applying in vitro and in vivo approaches regarding he effects of polyphenol-rich vegetables have been conducted. Most of these human intervention studies have been performed concerning the dietary intake of an ingredient, whereas many in vitro studies have been performed regarding individual polyphenol food components [3,4,11,13].

3. Bioavailability and Dietary Polyphenol Intake

Some studies used as references concerning daily polyphenol intake have demonstrated high variability in polyphenol consumption. A comprehensive survey of the food occurrence of polyphenols must be undertaken using well-standardized methods, and the content of each polyphenolic compound should be expressed as the amount provided by a food serving. A very useful tool is the Phenol-Explorer database to estimate the content of polyphenol in different food matrices. However, polyphenol intake depends not only on individual food preferences, but also on cultural and climate factors, which may influence the dietary consumption of distinct populations.

The mean intake of 377.5 mg per day of total polyphenol (distributed in 284.8 mg/day of phenolic acids and 54.6 mg per day of flavonoids) for people living in the biggest metropolitan area in Brazil, São Paulo has been estimated. Coffee contributed to 70.5% of total polyphenols (flavanols and

hydrocynnamic acids), citrus fruits, 4–6% and tropical fruits, 3–4% (flavonones and antocyanins). Intakes were higher in the elderly adults than in other adult groups ($p < 0.001$) and higher in individuals with lower educational levels [28]. In a subsequent study, where 620 elderly Brazilians participated in the survey, the average total polyphenol intake was of 1198.6 mg per day, distributed between phenolic acids (729.5 mg per day) and flavonoids (444.7 mg per day). The main dietary contributors for total polyphenols were coffee (45.8%) and beans (32.8%) [29]. The total polyphenol intake described for the elderly is similar to that estimated by a cohort study (1937 individuals (adults over the age of 18) of an urban population of Catania, Italy), where the dietary intake and major food sources of polyphenols in the Mediterranean healthy Eating, Aging and Lifestyles (MEAL) were evaluated. The mean polyphenol intake was of 663.7 mg per day; the most abundant classes were phenolic acids (362.7 mg per day) and flavonoids (258.7 mg per day), where the main dietary sources of total polyphenols were nuts, followed by tea and coffee, fruits, vegetables, chocolate, red wine and pasta [30].

Flavonol comsumption has been estimated at 20–25 mg per day and 35 mg per day in the USA, Denmar, Holland and Italy [31]. Europeans and Americans consume low amounts of isoflavones per day because of the very low consumption of soybeans in these countries. However, Asian isoflavones dietary ingestion is about 25–40 mg per day [32].

Studies concerning the bioavailability of polyphenols in several food matrices should be carried out to better understand the performances of these substances in the organism. The digestion, absorption and metabolization of each polyphenol differ, and there is frequently no relation between the polyphenol amount in food and the bioavailability in body. In the same way, the chemical structure of polyphenols in food matrices can be distinct from those found in human body fluids. For example, while aglycones are well absorbed in the small intestine, other polyphenols are well absorbed in other parts of the digestive tract. However, the majority of polyphenols arein the ester, glycoside and polymer forms and cannot be absorbed in their native form. These compounds must be hydrolyzed by enzymes from the colon microflora before being absorbed. For example, most glycoside forms resist acid hydrolysis in rat stomachs, although absorption in the gastric portion occurs for some flavonoids, such as quercetin, but not their glycosides [33]. In general, plasma concentrations vary according to the nature of the polyphenols and the food source [34,35].

During polyphenol absorption, chemical modifications, such as methylation, alkylation, sulfatation and glucuronidation, may take place in the small intestine and liver. Polyphenol methylation generally occurs at the C3 or C4 positions, and methylated polyphenols have been observed in plasma and urine [36]. In the liver, sulfo-transferase enzymes catalyze the transfer of a sulfate in the sulfation process that triggers metabolic activation [37]. The glucuronidation process occurs in the intestine and liver, where the highest conjugation rate is observed at the C3 position with a high efficiency rate. These chemical modifications restrict potential toxic effects and facilitate polyphenol biliary and urinary elimination. Extensively conjugated metabolites are preferentially eliminated in bile, while small conjugated metabolites are excreted through the urine. When polyphenols are secreted via the biliary route into the duodenum, bacterial enzymes act on these compounds causing their reasbsorption. In fact, the polyphenol forms that reach the blood and tissues may be distinct from the original forms found in foods. Thus, these chemical modifications add to the difficulties regarding the identification and evaluation of the biological activities of polyphenol metabolites [38].

4. Polyphenols and Their Cellular Effects

Phenolic compounds are related to a broad spectrum of medicinal properties with confirmed effects such as anti-allergic, anti-inflammatory, antibacterial, anti-thrombotic, vasodilators and, mainly, antioxidants [39]. Flavonoids, as other polyphenols, are important antioxidant compounds that can scavenge negative oxygen ions and free hydroxyl radicals in vivo [40].

Alongside the consumption of high amount of fruits and vegetables, that can impact human health [3], the beneficial effects derived from phenolic compounds found in these foods have been attributed to their antioxidant activity [41].

Phenolic acid benefits have also been exploited as cosmetic ingredients. Hydroxycinnamic acids and their derivatives have emerged as multifunctional ingredients for topical applications, since they display antioxidant, anti-collagenase, anti-inflammatory, antimicrobial and anti-tyrosinase activities, as well as ultraviolet (UV) protective effects, suggesting that they can be exploited as anti-aging and anti-inflammatory agents, preservatives and hyperpigmentation-correcting ingredients [42].

Commercially available ferulic and caffeic acids display anti-collagenase and photoprotection bioactivity. The use of 15–30 µM of ferulic acid and 3.75–30 µM caffeic acid promote the suppression of UVA-induced MMP-1 activity, offering protective activity to UVB-induced skin erythema [43].

Among the main vegetable products with high phenolic compound content, chocolate has been increasingly reported as a healthy and functional food, and the chocolate manufacturing industry has influenced the increase in the demand for this product [44].

5. Vegetables Popularly Consumed in Brazil as Polyphenol Sources

Vegetables are considered important sources of bioactive compounds, favoring human health and good organ function [45]. When the intake of foods rich in bioactive compounds is low, an increase in the production of reactive oxygen species (ROS) may occur, causing oxidative stress. Excess ROS may cause damage to DNA molecules, cell membranes, lipids and proteins [46]. This oxidative stress may lead to increased risks of developing non-communicable chronic diseases [47]. In addition, inadequate storage conditions and inappropriate culinary preparations may also adversely affect polyphenol content in different vegetables [48].

Brazil is among the five largest food producers in the world, producing several different crops of edible plants, for both domestic consumption and exportation [49]. Some of these vegetables that display health properties have been studied following several research interests. This review reports both the composition and the content of phenolic compounds found in commercial vegetables commonly consumed by the Brazilian population, such as taro corms and leaves, cassava, beetroot, soybeans and cocoa nibs. The polyphenol typesand contents found in each food matrix are listed in Table 1, Figure 5, and in the subsequent sections of this review.

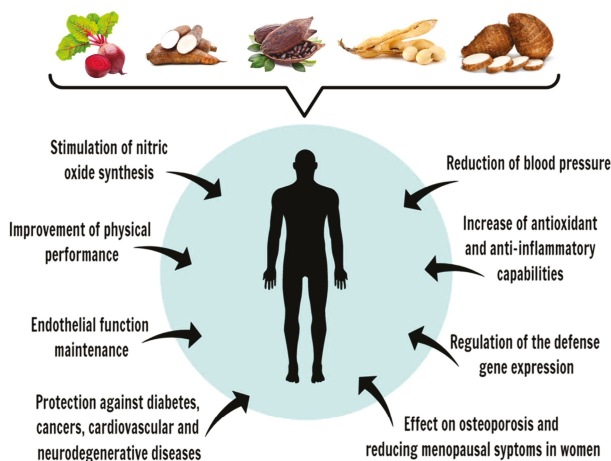


Figure 5. Polyphenolsfrom vegetables popularly consumed in Brazil—taro, beetroot, cassava, soybean and cocoa—and their effects on human health promotion and diseases.

Table 1. Flavonoid and non-flavonoid compounds plant sources.

Plant Source	Polyphenols	Class	Compound	Ref.
Beetroot (***B. vulgaris***)	flavonoids	flavanone	betagarin	[50]
		flavone	cochliophilin a	[50]
		flavonol	dihydroisorhamnetin	[50]
		isoflavone	betavulgarin	[50]
	non-flavonoids	hydrobenzoic acids	*n-trans*-ferruloylhomovanillylamine, *n-trans*-ferruloyltyramine	[50]
		hydroxycinnamic acids	caffeic acid, ferulic acid, gallic acid, *p*-cumaric, *p*-hydroxybenzoic, syringic acid, vanillic acid	[51]
Cassava (***M. esculanta***)	flavonoids	anthocyanidins	cyanidin, delphinidin	[52]
		flavan-3-ols	catechin, gallocatechin	[53]
		flavonols	kaempferol, quercetin, rutin	[54–57]
	non-flavonoids	coumarins	scopoletin	[58]
		hydrobenzoic acids	coniferaldehyde, gallic acid, isovanillin, syringsledehyde, resveratrol	[53,58]
		hydroxycinnamic acids	chlorogenic acid, *p*-coumaric acid	[53,58]
		lignans	balanophonin, pinoresinol	[58]
		stilbene	*trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene	[59]
Cocoa nibs (***T. cacao***)	flavonoids	anthocyanidins	arabinosidil, cyaniding, galactosidyl	[60,61]
		flavan-3-ols	cathechin, epicatechin, hyperoside, isovitexin, procyanidin b1, procyanidin b2, vitexin	[62]
		flavonols	quercetin, quercetin 3-*O*-arabinoside	[62]
		flavonones	apigenin, luteolin, luteolin-7-*O*-glucoside	[60]
		tannins	procyanidins	[60]
Soybean (***G. max***)	flavonoids	anthocyanidins	cyanidin, delphinidin, pelargonidin, petunidin	[63]
		hydrobenzoic acids	gallic acid, gentistic, protocatechuic acid	[64,65]
	non-flavonoids	hydroxycinnamic acids	caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, *p*-coumaric acid, *t*-cinnamic acid	[65]
		isoflavonoids	β-glucosides: daidzin, genistin, glycitin malonyl-β-glucosides: malonyldaidzin, malonylgenistin, malonylglycitin acetyl-β-glucosides: acetyldaidzin, acetylgenistin, acetylglycitin aglycones: daidzein, glycitein	[64,65]
Taro (***C. esculenta***) (corms/leaves)	flavonoids	anthocyanidins	cyanidin, delphinidin	[66]
		flavonols	isorhamnetin, kaempferol, myricetin, quercetin	[66]
	non-flavonoids	hydroxycinnamic acids	chlorogenic acid, *p*-coumaric acid	[67]

5.1. Beetroot (Beta vulgaris sp.)

Red beetroot has a high nutrition value (high sucrose content) and is considered a source of bioactive compounds, such as nitrate (NO_3^-), antioxidant substances and phenolic compounds, in addition to being a good source of dietary fiber, minerals (potassium, sodium, iron, copper, magnesium, calcium, phosphorus and zinc) and vitamins (retinol, ascorbic acid and B-complex) [66–68]. The red beetroot *Beta vulgaris* species is member of the Chenopodiaceae family, originated in regions of Europe and North Africa, where it is cultivated in mild to cold temperatures ranging from −10 to 20 °C. This plant species develops better in soils rich in organic matter and, therefore, the concentration of bioactive constituents depends on the plant development stage [69].

The antioxidant potential activity of red beetroot and its potential bioavailability in humans have been previously described [70]. The cited study reported that the most important betalain found in beetroot, betanin, is the most effective lipid peroxidation inhibitor, while other important compounds found in beetroot are polyphenols. A great controversy regardingpolyphenol contents in red beetroot exists, as in other vegetables. Indeed, in general, polyphenol content in vegetables is quite variable and can be characteristic for each genetic variety, but several factors like pedoclimatic (soil type, sun exposure and rainfall) or agronomic (culture in greenhouses or fields, biological culture and hydroponic culture) environmental factors, ripeness at the time of harvest, processing, and storage can affect the concentration of polyphenolic compounds in vegetables [71,72]. The total polyphenol content in beetroot has been reported as ranging from 720 to 3764 mg per kg [73,74].

A particular study determined the antioxidant potential, identified and quantified phenolic compounds in a juice prepared from beetroot, including gallic (ranging 10.8 to 30.4 mg L^{-1}), syringic (ranging 0.67 to 3.54 mg L^{-1}), caffeic (ranging 3.03 to 10.3 mg L^{-1}) and ferullic (ranging 0.25 to 1.24 mg L^{-1}) acids [44]. Considering a beetroot juice shot, the total polyphenol content ranged from 977.2 ± 5.2 to 1450.3 ± 42.1 mg and 3189.1 ± 77.3 to 1527.1 ± 18.0 mg of gallic acid equivalents/L before and after in vitro digestion, respectively [69]. Beetroot also displays antioxidant capacity estimated from ORAC and FRAP measurements, ranging from 23.9 to 37.9 mmol L^{-1} and 17.4 to 37.1 mmol L^{-1}, respectively, but the antioxidant capacity of beetroot juice differs among the beetroot varieties and is dependent on the total polyphenol content of each genetic variety analyzed [50]. Besides phenolic amides, such as *N-trans*-feruloyltyramine and *N-trans*-feruloylhomovanillylamine, and flavonoids such as betagarin, betavulgarin, cochliophilin A and dihydroisorhamnetin, several betalains, including vulgaxanthins I and II, betanin and isobetanin, have also been detected and quantified in red beetroot [75].

Although whole beetroot has become the most common formulation used for dietary administration, other beetroot formulations have been developed, such as beetroot juice and chips powder, and their antioxidant power and phenolic content has been evaluated [76,77]. Since foods in gel form might be the most effective formulation, due to the high concentration of bioactive compounds in a reduced volume, a beetroot gel has also been developed [78] from a concentrated beetroot juice and, as expected, showed higher levels of total phenolic compounds and antioxidant capacity, as well as other bioactive compounds [78]. The new beet formulation was shown to be easy to carry, ingest and tolerate, and easy to store at room temperature [79].

5.2. Cassava (Manihot esculenta)

Cassava is a tuberous woody shrub belonging to the Euphorbiaceae (spurge) family. This all-season crop, originated in the New World, but, is currently a staple food and animal feed in tropical and subtropical Africa, Asia and Latin America [80]. It is known as "tapioca" in Asian countries, as "mandioca", "aipim", "castelinha" and "macaxeira" in Brazil, as "yuca" in Spanish-speaking countries of Latin America, and as "manioc" in French-speaking countries in Africa [81].

Brazil is the second largest cassava producer in the world, with a production of 49% concentrated in Northeastern Brazil. In this region, skewed land distribution and semi-arid conditions go hand in hand with high poverty levels. According to the Brazilian Institute of Geography and Statistics [49],

the cassava-planted area in Brazil is of 2.035 million hectares, with a production of 23.242 million tons and an average yield of about 12.984 tons per ha [49]. Brazil's contribution to the world's cassava production is of around 16.2% and, for Latin America, of 77% [82]. In Brazil, two distinct cassava crops are available: *bitter (mandioca)* and *sweet (aipim)*. The bitter type is the main crop, with high cyanogenic potential, and should be processed prior to consumption. The main product is coarse, toasted flour used to complement many other dishes, enriching carbohydrate food content. Brazil has established a research program at the National Cassava and Fruit Research Center (CNPMF) that holds the world largest collection of cassava germplasm [49].

Cassava starch can be spontaneously fermented and sundried, and the resulting sour cassava starch ("polvilho azedo", in Brazil or "almidón agrio", in Colombia) is used for the production of gluten-free breads and biscuits that are very popular in some South American countries, and is considered the main source of dietary food energy for the majority of people living in the lowland tropics, and much of the subhumid tropics of West and Central Africa [83–85]. Certain cassava flour and starch properties, such as physical, chemical, physicochemical, pasting, and thermal parameters are important for its use in the food industry [84,86].

The phyto-nutritional status of cassava crops is influenced by several factors, such as plant genotypic background, climatic conditions, cultural practices and the use of inorganic fertilizers, causing significant variations in vitamin concentrations, antioxidant capacity and phenolic content [87].

Cassava roots can be processed into a wide variety of granules, pastes or flour, or consumed freshly boiled or raw. Processing can affect the nutritional value of cassava roots through molecule modifications and nutrient losses. Analyses performed on nutrient retention for each edible cassava product indicate that raw and boiled cassava root maintain the majority of their high-value nutrients, except for riboflavin and iron. Although raw cassava roots contain significant vitamin C amounts, they are very sensitive to heat and this nutrient can easily leach into water. Therefore, almost all of the processing techniques seriously affect raw cassava root content [88].

Cassava plant extracts contain a wide range of phenolic compounds including flavonoids, tannins, terpenoids, glycosides and alkaloids [86,89]. Cassava roots easily deteriorate during storage soon after their harvest and when, damaged by cutting or fungal infection, phenolic compounds, such as scopolin, scopoletin, and diterpenoids, are accumulated in the injured or infected regions [56,90,91]. The raw cassava tuber also contains alkaloids, flavonoids, tannins, reducing sugars and anthocyanosides, but does not contain cardiac glycosides, anthraquinone, phlobatinnins or saponins [88].

Coumarins are by far the main cassava component, while other polyphenols, like catechins, are present in minor concentrations and have been proven present in experimentally injured root parenchymas [92,93]. Among coumarins, catechin gallate, gallocatechin and flavone 3-glycosides (rutin and kaempferol 3-rutinoside) are observed, which have been suggested as displaying cardiovascular health benefits [56].

In addition, cassava also contains stilbenes. Two of these compounds, *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene and *trans*-3,4′,5-tetraxydroxystilbene (resveratrol), have been identified to date [59]. In addition, some unique compounds with a spiro configuration have also been isolated and characterized, such as the spiro biflavonoid larixinol, biosynthesized by combining two C15 units from a flavonoid origin and a number of novel spiro-structures, which were given the common names of yuccaols A–E [94,95], and a stilbenic portion closely related to resveratrol. The content of these stilbenes is described in Table 1 and have been compared to reverastrol content in the same matrix [95].

Cassava also contains several antioxidants, such as alpha- carotene and vitamin A [96,97]. In addition, cassava roots are both consumed fresh and stored for several days. An evaluation of the bioactive compounds present in this vegetable was carried out and reported the negative effects of its rapid deterioration [90,93,98]. Cassava tubers have been shown to contain polyphenol oxidase isozymes, which can oxidize monophenols into o-diphenols and/or diphenols into their corresponding o-quinones [99]. Cassava leaves present great potential for human and animal consumption as a green vegetable, in replacement of conventional protein sources; although it is important to note that cassava

leaves contain major anti-nutrients, such as hydrocyanic acid (HCN), tannic acid and phytic acid. Cassava leaves can provide proteins and vitamins A, B and C, as well as several minerals, including Mg, Fe, Zn and Mn [100,101]. In addition, anthocyanidins (cyanidin and delphinidin) [102] and flavonoids (quercetin, rutin and kaempferol) have also been identified in this cassava portion [103–105]. Tannins have also been reported, and their content in cassava leaves increases with plant maturity [106]. Shredding and sun-drying processing can reduce cyanide levels of cassava leaves, and may be associated with other methods in order to eliminate anti-nutrient compounds [96].

The selection of the solvent system significantly influences the recovery of each phenolic compound from cassava extracts. Phenolic compound content after an ethanol/acetone extraction was very similar for distinct cassava cultivars: gallic acid, 330 ± 4.5 mg kg^{-1}; gallocatechin, 91.0 ± 2.7 mg kg^{-1}; catechin, 15.6 ± 1.4 mg kg^{-1} and chlorogenic acid, 10.5 ± 3.32 mg kg^{-1} [53]. The anthocyanin content from cassava leaf stalks ranged from 44 mg g^{-1} in an acidified methanol extract, to 15 mg g^{-1} after acetone extraction. Antioxid antanthocyanin properties arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radicals to stabilize and delocalize unpaired electrons, as well as from their ability to chelate transition metal ions [107]. In addition, cassava flavonoid extracts display different health benefits [88,108,109]. In one study, ten antioxidant compounds (coniferaldehyde, isovanillin, 6-deoxyjacareubin, scopoletin, syringaldehyde, pinoresinol, *p*-coumaric acid, ficusol, balanophonin and ethamivan) were isolated and identified from cassava stems by an activity-guided isolation and were found to have DPPH scavenging capacity and ABTS free radical scavenging ability [58,110].

5.3. Cocoa Beans (Theobroma cacao) and Cocoa-Based Products

The fruit of the cocoa tree belonging to the Sterculiaceae family is a pod originated from Central America, widely cultivated by the Mayan civilization [111,112]. Cocoa is an important manufacturing plant with a significant influence on the economy of several countries [113] and is also a popular drink crop around the world, after coffee and tea. It is also widely used as the main ingredient in chocolate [114]. Chocolate is obtained from the fermented, roasted and milled seeds of the cocoa fruit by blending chocolate liquor with other ingredients, such as cocoa butter, sucrose, condensed milk or milk proteins, nuts, cereals or other ingredients [115].

Cocoa seeds are subdivided into cotyledon and embryo, protected by a pulp and a seed coat. They are basically constituted of 54% fat, 1.5% theobromine and caffeine, and polyphenol content represents 12 to 20% of the dry weight of the whole seed [116]. Polyphenol compounds are stored in the pigment cells of the cotyledons and are responsible for imparting bitterness and astringency to the cocoa fruit [117]. The natural microbiota involved in cocoa seed fermentation has a significant influence on polyphenol diffusion, but at the same time, is responsible for reducing almost 20% of the amount of these compounds in the final product [118]. Due to the high amount of polyphenols in cocoa, the number of studies demonstrating beneficial health effects of moderate consumption of phenolic compounds from cocoa products has increased. Improvementsin endothelial function, blood pressure, and cholesterol levels support potential benefits on cardiovascular function [119,120].

Spontaneous fermentation is a critical step for the development of chocolate flavor and taste, due to the generation of aroma precursors and alteration of the phenolic content of cocoa beans [121]. Bacteria and yeast consortia act on cocoa seeds during the fermentation process, producing indispensable metabolites, influenced by pH, temperature and oxygen availability [122]. During fermentation, the microbiota generates metabolites that influence flavor and may determine the quality of chocolate and cocoa-based products. During the fermentation process the production of lactic acid, acetic acid, lipids and proteins occurs, as well as of phenolic compounds that exert beneficial effects on human health.

The influence of phenolic compounds on chocolate flavor and color has been demonstrated in many studies, through reactions between polyphenols, sugar and amino acids, while alkaloids contribute to the bitterness of the fermented beans. Many studies have estimated phenolic compound contents during the fermentation process, where the highest total phenolic content was found after

24 h of fermentation, but decreased with longer fermentation times [123]. Polyphenols tend to diffuse out from the pigment cell of the cotyledons at the beginning of the fermentation process and undergo oxidation and complexation, producing mostly insoluble tannins. Polyphenol oxidases convert polyphenols into quinones, which then complex with proteins and peptides [118].

Of the several phenolic compounds identified and measured in cocoa beans and their by-products, such as cocoa liquor, cocoa powder and dark chocolate, the main groups are catechins or flavan-3-ols (37%), procyanidins (58%) and anthocyanins (4%) [115]. The main catechins are (−)-epicatechin, present in up to 35%, and, in smaller amounts, (+)-catechin, as well as traces of (+)-galloctechin and (−)-epigallocatechin. The anthocyanin fraction consists mainly of cyanidin-3-α-L-arabinosid and cyanidin-3-β-D-galactosid. Procyanidins comprise mostly flavan-3-4-diols, that are 4-8- or 4-6-bound to condensed dimers (procyanidin B1 dimers (166 mg kg^{-1}) and procyanidin B2 (921 mg kg^{-1}), trimers or oligomers with epicatechin (3.28 g kg^{-1}) as the main extension sub-unit [124]. In addition, cocoa also contains other polyphenols in minor amounts, such as quercetin (25 mg kg^{-1}), quercetin-3-*O*-glucoside (110 mg kg^{-1}) (isoquercitrin), quercetin-3-*O*-galactoside (90 mg kg^{-1}) (hyperoside), quercetin-3-*O*-arabinoside (165 mg kg^{-1}), as well as the following flavones: apigenin (5 mg kg^{-1}), apigenin-8-*C*-glucoside (4 mg kg^{-1}) (vitexin), apigenin-6-*C*-glucoside (4 mg kg^{-1}) (isovitexin), luteolin (5 mg kg^{-1}), luteolin-7-*O*-glucoside (12 mg kg^{-1}) and methylxanthines, mainly theobromine, as well as caffeine in small amounts [125].

Many studies have demonstrated the beneficial effects of chocolate on the cardiovascular system [126,127], where polyphenol have been shown to attenuate intracellular pro-inflammatory reactivity [128]. In obese and overweight individuals, dark chocolate-polyphenols were equally effective in reducing fasting blood glucose levels, systolic and diastolic blood pressures, while a decrease in free urinary cortisone levels in both groups was also observed [129]. Cocoa flavonols are effective in reducing blood pressure, insulin resistance, lipid peroxidation and improving cognitive function in mildly impaired elderly subjects [130]. High-quality dark chocolate is also the richest in theobromine and caffeine, which are vasodilator, diuretic and heart stimulant compounds [122].

5.4. Soybeans (*Glycine max*)

Soy grain is a polyphenol-rich legume belonging to the oilseed family, and its production is the highest among the grain legumes in the world [131]. The worldwide interest in this grain is due to the versatile usage of its derived products for bothhuman and animal consumption, which confers a high economic value to this grain in the national and international markets. Brazil is among the largest soybean producers in the world, since this grain legume is grown in several regions of the country [132].

Soybean is widely accepted as a healthy food, due to its nutritional composition and pharmacological effects, which have been attributed to the presence of several phenolic compounds. In this grain, polyphenols confer color, taste and sensory properties, such as sweetness, bitterness and astringency [4,133]. The concentration of polyphenols in soybeans is influenced by many factors, such as cultivar type, agricultural period, planting site, soil nutrition and shelf-life [64].

Soybean is rarely consumed simply by cooking the beans. Traditionally, it is processed to generate grain-derived products or ingredients for the food industry. Several soybean-derivatives are obtained by fermentation, such as tempeh and doulchi, or by coagulation only, such as several types of tofu [133]. In addition, soybeans can be used in the production of isolated proteins or hydrolyzed vegetable protein, which in turn are used as ingredients in meat and bakery products, beverages, soups and several other food products [134,135]. In these cases, soy proteins may confer texture, water retention and gelification, while increasing the polyphenol content of the processed food.

Soybeans contain isoflavones, anthocyanins, phytic acids, saponins, phenolic acids, hydroxybenzoic and hydroxycinnamic acids, isoflavonoids and anthocyanins [65]. Isoflavones possess high antioxidant activity and metal-ion chelating properties, but their major importance is their role as phytoestrogens, binding to estrogen receptors, and causing an estrogenic or anti-estrogenic effects

according to the type of estrogen receptor. Their intake has been associated with a decreased risk of hormone-related cancers [133].

Soybean phytoestrogens present low molecular masses and occur mainly in 4 chemical forms, malonyl-β-glucosides (70–80%), acetyl-β-glucosides (5%), β-glucosides (25%) and aglycones (2%), structurally similar to estrogen, estradiol-17β, and are able to activate or block estrogen effects, depending on the type of receptor, as mentioned previously [64,65,136].

The most investigated isoflavones are the aglycones daidzein and genistein, because they present antiproliferative activity against human breast cancer cell lineages by reducing the expression of estrogen receptors ERα and c-erbB-2 [137]. Phytoestrogens can bring many other health benefits besides the decreased risks of hormone-related cancers [138]. These include the prevention of vascular diseases through the inhibition of in vitro oxidation of the low-density lipoprotein involved in the pathogenesis of atherosclerosis [139], hypercolesteremia [140], osteoporosis [141] and menopausal symptoms [142].

Daidzein is mainly metabolized to equol and O-desmethylangolensin (O-DMA) by the human gut microflora, but both show strong biological activity [143]. In addition to isoflavones, black soybean possesses anthocyanins located in the soy seed coats, conferring the dark grain pigmentation [144]. The main anthocyanins found in black soybeans are cyanidin-3-*O*-glucoside, delphinidine-3-*O*-glucoside, and petunidin-3-*O*-glucoside [145], that display several biological activities, like antioxidant, anti-inflammatory, nephroprotective, antidiabectic, anticancer, anti-infertility, anti-obesity, anti-arthritic, neuroprotective, antihyperlipidemic and anti-cataract activity, as well as healing properties [63].

5.5. Taro (Colocasia esculenta)

C. esculenta, popularly known as taro, is an herbaceous plant native to India that belongs to Araceae family and is cultivated in both tropical and subtropical regions [146]. *C. esculenta* is capable of developing under conditions considered adverse to other species, such as poor soils, long raining periods, shading and other climatic stresses. This rustic plant displays low-cost crop production, with a high yield per unit area, is easy to maintain and resistant to pests and diseases [147,148].

Its edible corm is the main reason why this species is considered the 14th cultivated vegetable/staple around the world [149]. Taro contains substantial starch and fiber contents that can supply energy and satiate consumers, and is considered an essential food crop for millions of people worldwide [150]. Just not only is the corm edible, but taro leafs, petioles, inflorescences are also consumed, as well as taro derived-products, some of them industrialized, that have become outstanding and traditional foods worldwide. In order to add value and maintain quality, post-harvested technologies are usually applied to taro food and products, such as boiling, roasting, baking, frying in oil, pasting, milling and pounding [150,151], manufacturing alternative meals such as baby food, taro flakes, taro bread, dried taro chips, taro flour, noodles and even alcoholic beverages. Petioles are usually cooked and leaves are used in sauces and stews, purees or soups, while inflorescences are consumed cooked or fried [87].

Taro also shows other applications, not only in food products, but also for industrial purposes, including animal feed, insecticides, biodegradable products, and as an alcohol matrix producer [146]. Apart from its nutritional value, taro has also been investigated for its biological properties, due to the presence of a diverse range of biologically active phyto constituents present in the plant, such as flavonoids, alkaloids, sterols, tannins, phytates, glycosides and other micronutrients [151,152].

The well-known benefits promoted by polyphenol compounds, such as anti-inflammatory, antioxidant, antiallergic, hepatoprotective and antiviral activities, coincide with the therapeutic properties exerted by taro [153].

Forty-one phenolic compounds in taro leaves extracts have been identified and the main compounds are flavones di-*C*-glycosides, comprising around 84 to 87% of the total phenolic compounds in this matrix [87,154]. Tannins, flavonoids, triterpenoids and sterols have also been described in two

distinct varieties of the plant, the "giant white" and "red" taros, during the same development stage. The phenolic content of both varieties was ca. 9 g kg^{-1} but differences in the type of compounds reinforced the diversity and different amounts of phenolic compounds among *C. esculenta* cultivars [155].

A recent study [156] addressed the total phenolic and flavonoid composition of taro corm, and observed that flavonoids are present at around one quarter of the total phenolic content, contradicting previous studies [157] that reported significantly higher differences in the phenolic content of taro corm. The main flavonoid found in taro corm listed in the USDA 2013 database is quercetin, at an average of 28.7 mg kg^{-1} of the edible taro corm portion [158]. Anthoncyanins have also been identified in higher amounts in corm skin (16 mg) when compared to both corm and petioles (3.29 mg in both vegetable parts) [159].

As mentioned previously, taro can be processed in different forms and prepared by various methods, while the concentration and availability of bioactive constituents can be modified during tuber processing by boiling and frying [158]. Flavonoid and alkaloid content in taro do not decrease when compared to the raw form following boiling, unlike frying. On the other hand, tannin contents significantly increased during boiling and frying processes. Apparently, each processing method can generate new bonds between taro phytochemicals and cell structures, leading to higher or lower phenolic cleavages.

The industrial processing of taro into powder, noodles and cookies may modify its phytochemical contents. Therefore, a study was conducted to analyze the composition of raw taro, when processed into powder, noodles and cookies, and determine the effects of processing taro [160]. Results showed that, in general, taro powder increased phenol, tannin, flavonoid and saponin contents, whereas all these polyphenols are decreased in noodles and cookies, and tannins, in particular, are no longer detected. Cell disruption and decompartmentalization of phenolic compounds occurs when taro noodles and cookies are exposed to high temperatures for a long period of time, promoting polyphenol oxidase denaturation. Peeling, cutting and slicing taro also induces a quick enzymatic oxidation that helps to reduce the levels of these natural antioxidants and may cause negative effects on the final food products [160].

6. Dietary Intake of Selected Plants or Their Polyphenols and Human Health in Diseases: Intracellular Targets and Molecular Mechanisms

In the last decade, there has been much interest in the potential health benefits of dietary plant polyphenols, mainly as antioxidants. Although the mechanisms of protective polyphenol effects on degenerative diseases are not yet fully understood, several studies strongly suggest that long-term consumption of diets rich in plant polyphenols offers protection against different diseases. The phenolic groups within the structure of polyphenol molecules can accept an electron and thus form a stable phenoxyl radical, disrupting chain oxidation reactions in cellular components and, thereby, increasing plasma antioxidant capacity. Indeed, after polyphenol-rich food or beverage consumption, plasma antioxidant capacity increases significantly [18].

The antioxidant effect by which polyphenols exert theirbeneficial properties appears to involve their interaction with cellular signaling pathways and related machinery that mediate cell function under both normal and pathological conditions [161]. The signaling pathways for chronic and degenerative diseases should have their own intermediates and determining how polyphenols interact with those intermediate from the signaling pathways is the challenge to understand the multiple protective effects of those antioxidants. Nuclear factor kappa B, endothelial nitric oxide synthase and angiotensin converting enzyme have been recently identified, which may partly explain potential beneficial cardiovascular effects of polyphenols [162,163].

The following table lists selected biological effects related to the consumption of the electable plants of this study (taro, cocoa, cassava, soy and beetroot) or their polyphenols, described in a context of relevance to human health (Table 2).

Table 2. Human intervention studies evaluating the effects of polyphenols or polyphenol-enriched foods on health and diseases.

Polyphenols Source	Polyphenol Content(s)	Experimental Population	Number of Volunteers	Duration (Days)	Effect(s)
Isolated soy protein containing moderate and high isoflavones concentration	Two dietary groups: −56 mg of isoflavones −90 mg of isoflavones	Hypercholesterolemic postmenopausal women	66	168	Increases HDL cholesterol, mononuclear cell LDL receptor mRNA, both bone mineral content and density in the lumbar spine after ingestion of two dietary groups decreases in non-HDL cholesterol after ingestion of the two dietary groups (56 and 90 mg of isoflavones) [164].
Genistein (soy phytoestrogen)	54 mg	Healthy and postmenopausal women (range 47–57 years)	90	364	Decreased excretion of pyridinium and deoxypyridinoline (PYR: −54 ± 10%; DPYR: −55 ± 13%) after 6 and 12 (PYR: −42 ± 12%; DPYR: −44 ± 16%) months of genistein administration. Increases in serum bone-specific ALP (B-ALP) and osteocalcin (bone Gla protein [BGP]) after 6 (B-ALP: 23 ± 4%; BGP: 29 ± 11%) and 12 (B-ALP: 25 ± 7%; BGP: 37 ± 16%) months of genistein administration. Furthermore, significantly increases in femur (femoral neck: 3.6 ± 3% and lumbar spine (3 ± 2%) bone mineral density (BMD) were observed [165].
Textured soy protein high in isoflavones (HI); Textured soy protein low in isoflavones (LI)	HI 21.2 mg of daidzein; 34.8 mg of genistein; LI 0.9 mg of daidzein 1.0 mg of genistein	Healthy men and women (range 19–40 years)	24	14	Decreased plasma 8-epi-PGF$_2\alpha$ after high-isoflavone dietary treatment (326 ± 32 ng L^{-1}) when compared to the low-isoflavone dietary treatment (405 ± 50 ng L^{-1}). The lag time for copper-ion-induced LDL oxidation was longer after high-isoflavone dietary treatment (48 ± 2.4 min) than low-isoflavone dietary treatment (44 ± 1.9 min). No changes in plasma malondialdehyde, LDL α-tocopherol, polyunsaturated fatty acids, and isoflavonoids after dietary treatments [166].
Cocoa supplementation (dark chocolate bar and cocoa powder drink)	651 mg of procyanidins	Healthy men and women (range 20 to 60 years)	25	42	Decreased LDL oxidizability (evidenced by a longer lag time, 101.0 ± 20.7 min) after cocoa supplementation compared with baseline (91.3 ± 18.0 min) and washout (96.4 ± 7.5 min). No changes in urinary F(2) isoprostane concentration and markers of inflammation including the whole-blood cytokines, interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha, high sensitivity C-reactive protein and P-selectin [167].
Cocoa drink	821 mg of total flavonoids	Healthy men and women (range 18–72 years)	27	35	Increased peripheral vasodilation after four days of cocoa drink ingestion. After five days of cocoa drink consumption, pulse wave amplitude exhibited a large additional acute response [168].
Cocoa drink	176 mg of flavan-3-ols (70 mg of epicatechin plus catechin and 106 mg of procyanidins)	Outpatients with at least 1 cardiovascular risk factor (means, 41 years)	26	2	Increased flow-mediated dilatation maximally at 2 h from 3.4% to 6.3% after cocoa drink ingestion. Increases nitrosylated and nitrosated species from 22 to 36 nmol L^{-1} after ingestion of cocoa rich in flavan-3-ols [169].
Dark chocolate	15.6 mg of epicatechin equivalents per gram	Heart transplant recipients volunteers (range 35–70 years)	22	Acute	Increased coronary artery diameter from 2.36 ± 0.51 to 2.51 ± 0.59 mm after ingestion of flavonoid-rich dark chocolate. Decreased platelet adhesion from 4.9 ± 1.1% to 3.8 ± 0.8% after ingestion of flavonoid-rich dark chocolate [170].
Powder cocoa drink	963 mg of flavonoids	Diabetes mellitus II men and women (for at least 5 years, range 50 to 80 years)	41	28	Increased flow-mediated dilatation (FMD) by 30% after ingestion of flavanol-containing cocoa. Treatment was well tolerated, without evidence of tachyphylaxia. No changes in endothelium-independent responses, blood pressure, heart rate, and glycemic control after ingestion of the cocoa drink [171].

It is evident that polyphenol compounds should be sufficiently absorbed, crossing the intestinal barrier and reaching micromolar concentrations in the bloodstream, where they have been shown to present biological effects [9,18]. However, most studies were not conducted in vivo, but in vitro, and did not take in account metabolic and bioavailability factors, while the reported observations do not necessarily occur in vivo. In general, polyphenol concentrations must range from 0.1 to 100 μmol L^{-1} in order to observe biological in vitroeffects. Taking into consideration that polyphenol physiological concentrations are less than 10 μmol L^{-1}; the effects of polyphenols assayed in vitro using concentrations above 10 μmol L^{-1} may not be valid. In addition, the polyphenol forms that appear in the blood may be different from those found in foods, due to the extensive metabolism suffered by these compounds in the human organism, as mentioned previously.

The organism is in a state of redox equilibrium in basal metabolic conditions, presenting a balance between oxidant and antioxidant agents. At low concentrations, oxidant agents originating from the cellular metabolism, such as reactive oxygen and nitrogen species (RONS) play important roles in several cellular and biochemical processes, including gene expression, cell proliferation, apoptosis and muscle contraction [172]. However, overexposure of a cell to RONS (as happens through exposure to infectious pathogens during metabolic and cardiovascular diseases, degenerative diseases, inflammatory processes or even in some physiological conditions, such as aging, can overwhelm the oxidant state, leaving DNA, carbohydrate, protein and lipid structures susceptible to oxidation and functional deficiencies, or, in other words, causing an imbalance in redox homeostasis and promoting oxidative stress [173].

LDL oxidation is considered one of the main mechanisms in atherosclerosis development, a chronic inflammatory disease that develops in lesion-prone regions of medium-sized arteries and can lead to the development of pathological conditions, which may result in death [38]. The total antioxidant activity before and after in vitro digestion procedure with simulated gastric and duodenal phases of beetroot juice shots [75] for example, has been evaluated, where an approximate 3-fold increase in total antioxidant capacity was observed, evaluated by ferric reducing antioxidant power (FRAP), after the gastric phase (33,731.42 $\pm$ 298.57 μmol L^{-1}), with a marked decrease after the duodenal phase (24,861.42 $\pm$ 301.42 μmol L^{-1}). The authors reported that the high antioxidant capacity of the beetroot juice shot was attributed to its polyphenol contents. The antioxidant capacity of 4880 $\pm$ 68.6 mg of GAE L^{-1} polyphenol content was reduced to 3188.6 $\pm$ 77.14 mg GAE L^{-1} following the gastric phase digestion, but still at 2/3 of the initial antioxidant level.

Cocoa polyphenol compounds display anti-inflammatory antioxidant and anti-atherogenic effects that promote several cardiovascular events. As expected for polyphenol, those present in cocoa polyphenols increase HDL-c (HDL-cholesterol), reduce LDL oxidation, inhibit platelet aggregation and decrease vascular cell adhesion, improving endothelial function and reducing blood pressure. But can also modulate intestinal inflammation through the reduction of neutrophil infiltration and expression of different transcription factors, which leads to decreases in the production of proinflammatory enzymes and cytokines.They also have antiproliferative, antimutagenic, and chemoprotective effects, in addition to their anticariogenic effects [174,175].

Yuccaols (A, B, C) are phenolic constituents isolated from *Yucca schidigera* barkm characterized by unusual spirostructures made up of a C15 unit and a stilbenic portion closely related to resveratrol, and are associated with the prevention of the platelet aggregation. This polyphenol inhibits cyclooxigenase 1 (COX 1) activity, reducing the synthesis of thromboxane A2 (TXA_2), a vasoconstrictor and platelet aggregation inducer.The anti-inflammatory properties attributed to Yucca schidigera can be ascribed to both resveratrol and Yuccaols and provide the first evidences of the anti-tumor and anti-invasive properties of these novel phenolic compounds [176].

On the other hand, isoflavone intervention studies are perhaps the more advanced of polyphenol effects in human beings. Several studies have demonstrated protective isoflavone effects on lipid peroxidation and increased LDL resistance to oxidation. These studies have also shown effects on bone biomarkers, such as increased serum concentrations of bone-specific alkaline phosphatase and

osteocalcin [165,177]. Bone metabolism and bone mineral density (BMD) were evaluated in 90 healthy post-menopausal women following the intake of the phytoestrogen genistein. Genistein prevented bone loss influenced by estrogen-deficiency without exerting negative effects on the uterus and breast. Furthermore, a decrease in bone resorption markers and enhanced bone formation parameters were observed. Genistein may exert these aforementioned beneficial effects on bones by inducing their apoptosis through the Ca^{2+} signaling pathway, and can also act directly by suppressing osteoclasts, decreasing the amount of these bone cells. This suppressing effect on osteoclasts may occur, at least in part, by the inhibition of protein kinase and the activation of protein tyrosine phosphatase caused by genistein. Furthermore, genistein significantly stimulates thymidine incorporation and increases cell numbers in human vertebrae–derived bone cells [165,177].

Atherosclerosis, a chronic inflammatory disease that develops in lesion-prone regions of medium-sized arteries, may be clinically silent for decades before becoming active and producing pathological conditions (acute myocardial infarction, unstable angina or sudden cardiac death). The effects of 17 days of a diet enriched in soy isoflavones containing 21.2 mg of daidzein and 34.8 mg of genistein were investigated on the in vivo biomarkers of lipid peroxidation and LDL resistance to oxidation, in 24 healthy volunteers (19 women and 5 men). The plasma concentration of 8-epi-prostaglandin F(2)(alpha) was decreased after the soy isoflavones treatment concomitant with the lag time extension for copper-ion-induced LDL oxidation [166].

The polyphenols may exert protective effects on human cancer cell lines, reducing the number or the growth of tumorigenic cells [178]. Polyphenols such as catechins, isoflavones, quercetin, flavanones, lignans, curcumin and resveratrol have been tested and all of them showed protective benefic effects in some tumor models although their action mechanisms are distinct [179]. Some chemoprevention mechanisms of these above mentioned polyphenols are changes in cellular signaling, induction enzymes detoxification, antiestrogenic activity, antiproliferation, anti-inflammatory activity, regulation of the host immune system, prevention of oxidation and induction of cell cycle arrest or apoptosis [175].

A study evaluated the effects of 1-month consumption of soy-derivative foods rich in isoflavonoid phytoestrogens (about 86 mg isoflavones) on LDL oxidation and sex hormone receptor activity in 31 hyperlipidemic subjects [180]. The authors observed a decrease in both oxidized LDL measured as conjugated dienes in the LDL fraction and the ratio of conjugated dienes to LDL cholesterol after consumption of soy-derivative foods rich in isoflavonoids. However, a non-significant decrease was detected in estrogenic and androgenic hormone activities, evaluated by estrogen and androgen (dihydrotestosterone) hormones after isoflavonoids-rich food intake. This result is important because it has already proven by scientific community that a massive increased estrogen levels tend to stimulate uncontrolled cell growth of breast epithelium, contributing to the development of breast cancer [181]. However, in the study mentioned above [180], no significant difference was detected in ex vivo estrogenic activities in urine samples of women after intake of high-isoflavones foods. Additionally, the reduction of the levels of circulating oxidized LDL was observed. Soybeans consumption may reduce cardiovascular disease risk without increasing the risk for hormone-dependent cancers, but confirming a tendency for the reduction of in ex vivo sex hormone activity. Soy-isoflavones ingestion could be administered in association with sex hormone blocking agents to treat and possibly prevent hormone-dependent cancers [181].

As mentioned before, catechin is a phenolic compound found at high concentrations in dark chocolate. Due to the widespread non-galloylated forms, it is difficult to estimate catechins during intervention studies. Catechins display many in vitro and in vivo effects. They can increase plasmatic antioxidant activity, the resistance of LDL to oxidation, raise ascorbate concentrations, decrease plasma lipid peroxide and malondialdehyde concentrations, and reduce non-heme iron absorption [182,183]. Several epidemiologic studies have demonstrated the beneficial association between cocoa intake and lower cardiovascular disease mortality [184,185], cardiovascular risk factors, such as lipid profile, insulin resistance and vascular dilatation [186–188].

The ingestion of increasing amounts of cocoa powder (13, 19.5 and 26 g day^{-1}) for 4 weeks altered LDL and oxidized LDL cholesterol concentrations in the plasma of normocholesterolemic and mildly hypercholesterolemic volunteers. The polyphenol ingestion, even in the lowest cocoa amount, was shown to contribute to the reduction in the plasmatic levels of LDL cholesterol, to the elevation of HDL cholesterol, and the suppression of oxidized LDL [189]. In another study, the ingestion of dark chocolate (100 g day^{-1} containing 70% of cocoa) for 7 days caused a significant increase in plasmatic HDL cholesterol concentrations and a non-significant decrease in LDL, total cholesterol concentrations, but showed favorable effects on inflammatory markers, confirming that polyphenol compounds from cocoa have contributed to beneficial effects on cholesterol metabolism [190].

Acute and sustained dose-dependent consumption of cocoa products have shown beneficial effects on endothelial function by FMD improvement [191], due to increases in plasmatic nitric oxide (NO) concentrations after cocoa beverage consumption in healthy subjects [192]. Cocoa polyphenols improve endothelial functions by increasing NO synthase (NOS) activity [168,169], leading to the inhibition of platelet adhesion and aggregation, and decrease of systolic and diastolic blood pressures [170].

7. Conclusions

Increasing evidence is available which indicates that the consumption of phenolic compounds present in natural foods may lower the risk of serious health disorders. The antioxidant power of polyphenols can effectively scavenge free radicals, absorb light in the UV region and chelate transition metals, among others. In addition to exerting protective effects, these compounds can also interact with intracellular signaling cascades, raising or activating transcriptional factors that impair the expression of several genes including, among others, COX_2, $_{i}$NOS, cyclins and NADP oxidase. Polyphenols can also act to regulate the activity of proteins and enzymes already synthetized in the organism. These molecular mechanisms, although not yet completely established, result in protection against chronic and degenerative diseases.

The polyphenols found in root and tubercle vegetables, such as taro, beetroot and cassava, and in grains, like soybean and cocoa, described herein are involved in preventing chronic and degenerative diseases.

Regarding cardiovascular diseases, these polyphenols seems to be able to alter the lipid metabolism by inhibiting LDL oxidation, reducing atherosclerotic lesions, inhibiting platelet aggregation, decreasing malondialdehyde concentrations and, consequently, improving endothelial function and reducing systolic and diastolic blood pressures. In cancer, polyphenols are involved in inhibiting cancer cell proliferation and reducing the number of tumorigenic cells. In menopausal health impairments and symptoms, polyphenols, particularly the isoflavones-phytoestrogens found in soybeans, have been shown to reduce in vivolipid peroxidation and increase LDL resistance to oxidation, which in turncan improve bone metabolism, bone mineral density andcan promote cardiovascular protection, as well asmimic estrogen-hormones.

The food products described herein, in raw form, show a high diversity of phenolic compounds, but the lack of bioaccessibility for each form of consumption must be known before the recommendation of these products as dietary supplements or functional food ingredients for health promotion and/or reduction of risk diseases. Dosage recommendations and frequency of intake should be better evaluated.

Moreover, natural polyphenols obtained from edible sources, by-products and co-products may display important applications in the food industry, where they can be used as dyes or food preservatives since they can arrest progressive food oxidation damage while avoiding the production of off-odors and off-flavors. Natural antioxidants from edible sources are considered safer alternatives for food preservation when compared to synthetic antioxidants, since they can avoid the adverse reactions of food additives to human health.

Acknowledgments: The authors would like to acknowledge financial support from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro).

Author Contributions: All authors contributed to the writing of the document and approved the final version of the review.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pérez-Jiménez, J.; Neveu, V.; Vos, F.; Scalbert, A. Identification of the 100 richest dietary sources of polyphenols: An application of the phenol-explorer database. *Eur. J. Clin. Nutr.* **2010**, *64*, S112–S120. [CrossRef] [PubMed]
2. Gupta, A.; Kagliwal, L.D.; Singhal, R.S. Biotransformation of polyphenols for improved bioavailability and processing stability. *Adv. Food Nutr. Res.* **2013**, *69*, 183–217. [PubMed]
3. Perez-Hernandez, J.; Zaldivar-Machorro, V.J.; Villanueva-Porras, D.; Vega-Avila, E.; Chavarria, A. A potential alternative against neurodegenerative diseases: Phytodrugs. *Oxid. Med. Cell Longev.* **2016**, *2016*, 8378613. [CrossRef] [PubMed]
4. Cheynier, V.; Tomas-Barberan, F.A.; Yoshida, K. Polyphenols: From Plants to a Variety of Food and Nonfood Uses. *J. Agric. Food Chem.* **2015**, *63*, 7589–7594. [CrossRef] [PubMed]
5. Grosso, G.; Micek, A.; Godos, J.; Pajak, A.; Sciacca, S.; Galvano, F.; Giovannucci, E.L. Dietary Flavonoid and Lignan Intake and Mortality in Prospective Cohort Studies: Systematic Review and Dose-Response Meta-Analysis. *Am. J. Epidemiol.* **2017**, *3*, 1–13. [CrossRef] [PubMed]
6. Menezes, R.; Rodriguez-Mateos, A.; Kaltsatou, A.; González-Sarrías, A.; Greyling, A.; Giannaki, C.; Andres-Lacueva, C.; Milenkovic, D.; Gibney, E.R.; Dumont, J.; et al. Impact of Flavonols on Cardiometabolic Biomarkers: A Meta-Analysis of Randomized Controlled Human Trials to Explore the Role of Inter-Individual Variability. *Nutrients* **2017**, *9*, 117. [CrossRef] [PubMed]
7. Wang, X.; Ouyang, Y.Y.; Liu, J.; Zhao, G. Flavonoid intake and risk of CVD: A systematic review and meta-analysis of prospective cohort studies. *Br. J. Nutr.* **2014**, *111*, 1–11. [CrossRef] [PubMed]
8. Liu, Y.J.; Zhan, J.; Liu, X.L.; Wang, Y.; Ji, J.; He, Q.Q. Dietary flavonoids intake and riskof type 2 diabetes: A meta-analysis of prospective cohort studies. *Clin. Nutr.* **2014**, *33*, 59–63. [CrossRef] [PubMed]
9. Middleton, E.; Kandaswami, C.; Theoharides, T.C. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673–751. [PubMed]
10. Vermerris, W.; Nicholson, R. Families of phenolic compounds and means of classification. In *Phenolic Compound Biochemistry*; Springer: Dordrecht, The Netherlands, 2006; pp. 1–34.
11. Quideau, S.; Deffieux, D.; Douat-Casassus, C.; Pouysegu, L. Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angew. Chem. Int. Ed. Engl.* **2011**, *50*, 586–621. [CrossRef] [PubMed]
12. Giada, M. Food phenolic compounds: Main classes, sources and their antioxidant power. In *Oxidative Stress and Chronic Degenerative Diseases—A Role for Antioxidants*, 1st ed.; Morales-Gonzále, J.A., Ed.; InTech: Rijeka, Croatia, 2013; pp. 87–112.
13. Weichselbaum, E.; Buttriss, J. Polyphenols in the diet. *Nutr. Bull.* **2010**, *35*, 157–164. [CrossRef]
14. Charrouf, Z.; Guillaume, D. Phenols and polyphenols from *Argania spinosa*. *Am. J. Food Technol.* **2007**, *2*, 679–683.
15. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*, 1231–1246. [CrossRef] [PubMed]
16. Neveu, V.; Perez-Jimenez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D. Phenol-explorer: An online comprehensive database on polyphenol contents in foods. *Database* **2010**, *2010*. [CrossRef] [PubMed]
17. Pereira, D.M.; Valentão, P.; Pereira, J.A.; Andrade, P.B. Phenolics: From chemistry to biology. *Molecules* **2009**, *14*, 2202–2211. [CrossRef]
18. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S. [PubMed]
19. Başkan, K.S.; Tütem, E.; Akyüz, E.; Apak, R. Assessment of the contributions of anthocyanins to the total antioxidant capacities of plant foods. *Eur. Food Res. Technol.* **2015**, *241*, 529–541. [CrossRef]

20. Araújo, M.M.; Fanaro, G.B.; Villavicencio, A.L.C.H. Soybean and isoflavones—From farm to fork. In *Soybean-Bio-Active Compounds*; El-Shemy, H.A., Ed.; InTech: Rijeka, Croatia, 2013; pp. 2–21.
21. D'Archivio, D.M.; Filesi, C.; Di Benedetto, R.; Gargiulo, R.; Giovannini, C.; Masella, R. Polyphenols, dietary sources and bioavailability. *Ann. Ist. Super. Sanita* **2007**, *43*, 348–361. [PubMed]
22. Shahidi, F.; Ambigaipalan, P. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects—A review. *J. Funct. Foods* **2015**, *18 Pt B*, 820–897. [CrossRef]
23. Wallace, T.C.; Giusti, M.M. Anthocyanins. *Adv. Nutr.* **2015**, *6*, 620–622. [CrossRef] [PubMed]
24. Ferrazzano, G.F.; Amato, I.; Ingenito, A.; Zarrelli, A.; Pinto, G.; Pollio, A. Plant polyphenols and their anti-cariogenic properties: A review. *Molecules* **2011**, *16*, 1486–1507. [CrossRef] [PubMed]
25. Ajila, C.; Brar, S.; Verma, M.; Tyagi, R.; Godbout, S.; Valero, J. Extraction and analysis of polyphenols: Recent trends. *Crit. Rev. Biotechnol.* **2011**, *31*, 227–249. [CrossRef] [PubMed]
26. Tomas-Barberan, F.A.; Andres-Lacueva, C. Polyphenols and health: Current stateand progress. *J. Agric. Food Chem.* **2012**, *60*, 8773–8775. [CrossRef] [PubMed]
27. Visioli, F.; Davalos, A. Polyphenols and cardiovascular disease: A critical summary of the evidence. *Mini Rev. Med. Chem.* **2011**, *11*, 1186–1190. [PubMed]
28. Miranda, A.M.; Steluti, J.; Fisberg, R.M.; Marchoni, D.M. Dietary intake and food contributors of polyphenols in adults and elderly adults of Sao Paulo: A population-based study. *Br. J. Nutr.* **2016**, *115*, 1061–1070. [CrossRef] [PubMed]
29. Nascimento-Souza, M.A.; de Paiva, P.G.; Pérez-Jiménez, J.; Franceschini, S.D.C.C.; Ribeiro, A.Q. Estimated dietary intake and major food sources of polyphenols in elderly of Viçosa, Brazil: A population-based study. *Eur. J. Nutr.* **2016**, *21*, 1–11. [CrossRef] [PubMed]
30. Godos, J.; Marventano, S.; Mistretta, A.; Galvano, F.; Grosso, G. Dietary sources of polyphenols in the Mediterranean healthy Eating, Aging and Lifestyle (MEAL) study cohort. *Int. J. Food Sci. Nutr.* **2017**, *68*, 750–756. [CrossRef] [PubMed]
31. Vogiatzoglou, A.; Mulligan, A.A.; Lentjes, M.A.; Luben, R.J.; Spencer, J.P.; Schroeter, H.; Khaw, K.T.; Kuhnle, G.G. Flavonoid intake in European adults (18 to 64 years). *PLoS ONE* **2015**, *10*, e0128132. [CrossRef] [PubMed]
32. Jun, S.; Shin, S.; Joung, H. Estimation of dietary flavonoid intake and major food sources of Korean adults. *Br. J. Nutr.* **2016**, *115*, 480–489. [CrossRef] [PubMed]
33. Kanimozhi, S.; Bhavani, P.; Subramanian, P. Influence of the flavonoid, quercetin on antioxidant status, lipid peroxidation and histopathological changes in hyperammonemic rats. *Indian J. Clin. Biochem.* **2017**, *32*, 275–284. [CrossRef] [PubMed]
34. Ding, F.; Peng, W. Biological activity of natural flavonoids as impacted by protein flexibility: An example flavanones. *Mol. Biosyst.* **2015**, *11*, 1119–1133. [CrossRef] [PubMed]
35. Perira-Caro, G.; Borges, G.; van der Hooft, J.; Clifford, M.N.; Del Rio, D.; Lean, M.E.; Roberts, S.A.; Kellerhals, M.B.; Crozier, A. Orange juice (poly)phenols are highly bioavailable in humans. *Am. J. Clin. Nutr.* **2014**, *100*, 1378–1384. [CrossRef] [PubMed]
36. Lee, M.J.; Maliakal, P.; Chen, L.; Meng, X.; Bondoc, F.Y.; Prabhu, S.; Lambert, G.; Mohr, S.; Yang, C.S. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: Formation of different metabolites and individual variability. *Cancer Epidemiol. Biomark. Prev.* **2002**, *11*, 1025–1032.
37. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [CrossRef] [PubMed]
38. Pandey, K.B.; Mishra, N.; Rizvi, S.I. Protective role of myricetin on markers of oxidative stress in human erythrocytes subjected to oxidative stress. *Nat. Prod. Commun.* **2009**, *4*, 221–226. [PubMed]
39. Yildrim, A.B.; Guner, B.; Karakas, F.P.; Turjer, A.U. Evaluation of antibacterial, antitumor, antioxidant activities and phenolic constituents of field-grown and in vitro- grown *Lysimachia vulgaris*. *Afr. J. Tradit. Complement. Altern. Med.* **2017**, *14*, 177–187. [CrossRef] [PubMed]
40. Eghbaliferiz, S.; Iranshahi, M. Prooxidant Activity of Polyphenols, Flavonoids, Anthocyanins and Carotenoids: Updated Review of Mechanisms and Catalyzing Metals. *Phytother. Res.* **2016**, *30*, 1379–1391. [CrossRef] [PubMed]
41. Singh, M.; Kaur, M.; Silakari, O. Flavones: An important scaffold for medicinal chemistry. *Eur. J. Med. Chem.* **2014**, *84*, 206–239. [CrossRef] [PubMed]

42. Taofiq, O.; González-Paramás, A.M.; Barreiro, M.F.; Ferreira, I.C. Hydroxycinnamic acids and their derivatives: Cosmeceutical significance, challenges and future perspectives, a Review. *Molecules* **2017**, *22*, 281. [CrossRef] [PubMed]
43. Pluemsamran, T.; Onkoksoong, T.; Panich, U. Caffeic acid and ferulic acid inhibit UVA-induced matrixmetalloproteinase-1 through regulation of antioxidant defense system in keratinocyte HaCaT cells. *Photochem. Photobiol.* **2012**, *88*, 961–968. [CrossRef] [PubMed]
44. Belščak-Cvitanović, A.; Komes, D.; Dujmović, M.; Karlović, S.; Biškić, M.; Brnčić, M.; Ježek, D. Physical, bioactive and sensory quality parameters of reduced sugar chocolates formulated with natural sweeteners as sucrose alternatives. *Food Chem.* **2015**, *167*, 61–70. [CrossRef] [PubMed]
45. Holst, B.; Williamson, G. Nutrients and phytochemicals: From bioavailability to bioefficacy beyond antioxidants. *Curr. Opin. Biotechnol.* **2008**, *19*, 73–82. [CrossRef] [PubMed]
46. Chen, H.; Yoshioka, H.; Kim, G.S.; Jung, J.E.; Okami, N.; Sakata, H.; Maier, C.M.; Narasimhan, P.; Goeders, C.E.; Chan, P.H. Oxidative stress in ischemic brain damage: Mechanisms of cell death and potential molecular targets for neuroprotection. *Antioxid. Redox Signal.* **2011**, *14*, 1505–1517. [CrossRef] [PubMed]
47. Singh, P.N.; Arthur, K.N.; Orlich, M.J.; James, W.; Purty, A.; Job, J.S.; Rajaram, S.; Sabaté, J. Global epidemiology of obesity, vegetarian dietary patterns, and noncommunicable disease in Asian Indians. *Am. J. Clin. Nutr.* **2014**, *100*, 359S–364S. [CrossRef] [PubMed]
48. Faller, A.L.K.; Fialho, E. The antioxidant capacity and polyphenol content of organic and conventional retail vegetables after domestic cooking. *Food Res. Int.* **2009**, *42*, 210–215. [CrossRef]
49. IBGE 2017—Produção Agrícola Municipal, 2017. Brazilian Agricultural Research Corporation. Ministry of Agriculture, Livestock, and Food Supply. Available online: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/ (accessed on 16 September 2017).
50. Kujala, T.S.; Vienola, M.S.; Klika, K.D.; Loponen, J.M.; Pihlaja, K. Betalain and phenolic compositions of four beetroot (*Beta vulgaris*) cultivars. *Eur. Food Res. Technol.* **2002**, *214*, 505–510. [CrossRef]
51. Wruss, J.; Waldenberger, G.; Huemer, S.; Uygun, P.; Lanzerstorfer, P.; Müller, U.; Höglinger, O.; Weghuber, J. Compositional characteristics of commercial beetroot products and beetroot juice prepared from seven beetroot varieties grown in Upper Austria. *J. Food Compos. Anal.* **2015**, *42*, 46–55. [CrossRef]
52. Uarrota, V.G.; Maraschin, M. Metabolomic, enzymatic, and histochemical analyzes of cassava roots during postharvest physiological deterioration. *BMC Res. Notes* **2015**, *5*, 648–663. [CrossRef] [PubMed]
53. Gogbeu, S.J.; Dogbo, D.O.; Gonnety, J.T.; N'zue, B.; Zohouri, G.P.; Boka, A. Study of some characteristics of soluble polyphenol oxidases from six cultivars callus of cassava (*Manihot esculenta* Crantz). *J. Anim. Plant Sci.* **2011**, *9*, 1169–1179.
54. Kubo, I.; Masuoka, N.; Nihei, K.; Brigitta, B. Maniçoba, a quercetin-rich Amazonian dish. *J. Food Compos. Anal.* **2006**, *19*, 579–588. [CrossRef]
55. Bayoumi, A.L.; Rowan, M.G.; Beeching, J.R.; Blagbrough, I.S. Investigation of biosynthetic pathways to hydroxycoumarins during post-harvest physiological deterioration in cassava roots by using stable isotope labelling soad. *ChemBioChem* **2008**, *9*, 3013–3022. [CrossRef] [PubMed]
56. Buschmann, H.; Rodriguez, M.X.; Tohme, J.; Beeching, J.R. Accumulation of hydroxycoumarins during post-harvest deterioration of tuberous roots of cassava (*Manihot esculenta* Crantz). *Ann. Bot.* **2000**, *86*, 1153–1160. [CrossRef]
57. Uarrota, V.G.; Nunes, E.C.; Peruch, L.A.; Neubert Ede, O.; Coelho, B.; Moresco, R.; Domínguez, M.G.; Sánchez, T.; Meléndez, J.L.; Dufour, D.; et al. Toward better understanding of postharvest deterioration: Biochemical changes in stored cassava (*Manihot esculenta* Crantz) roots. *Food Sci. Nutr.* **2015**, *4*, 409–422. [CrossRef] [PubMed]
58. Yi, B.; Hu, L.; Mei, W.; Zhou, K.; Wang, H.; Luo, Y.; Wei, X.; Dai, H. Antioxidant phenolic compounds of cassava (*Manihot esculenta*) from Hainan. *Molecules* **2011**, *16*, 10157–10167. [CrossRef] [PubMed]
59. Cheeke, P.R.; Piacente, S.; Oleszek, W. Anti-inflammatory and anti-arthritic effects of *Yucca schidigera*: A review. *J. Inflamm.* **2006**, *3*, 6. [CrossRef] [PubMed]
60. Efraim, P.; Alves, A.B.; Jardim, D.C.P. Polyphenols in cocoa and derivatives: Factors of variation and health effects. *Braz. J. Food Technol.* **2011**, *14*, 181–201. [CrossRef]
61. Khan, N.; Khymenets, O.; Urpí-Sardà, M.; Tulipani, S.; Garcia-Aloy, M.; Monagas, M.; Mora-Cubillos, X.; Llorach, R.; Andres-Lacueva, C. Cocoa polyphenols and inflammatory markers of cardiovascular disease. *Nutrients* **2014**, *6*, 844–880. [CrossRef] [PubMed]

62. Ortega, N.; Romero, M.P.; Macià, A.; Reguant, J.; Anglès, N.; Morelló, J.R.; Motilva, M.J. Obtention and characterization of phenolic extracts from different cocoa sources. *J. Agric. Food Chem.* **2008**, *56*, 9621–9627. [CrossRef] [PubMed]
63. Ganesan, K.; Xu, B. A critical review on polyphenols and health benefits of black soybeans. *Nutrients* **2017**, *9*, 455. [CrossRef] [PubMed]
64. Riedl, K.M.; Lee, J.H.; Renita, M.; St Martin, S.K.; Schwartz, S.J.; Vodovotz, Y. Isoflavone profiles, phenol content, and antioxidant activity of soybean seeds as influenced by cultivar and growing location in Ohio. *J. Sci. Food Agric.* **2007**, *87*, 1197–1206. [CrossRef]
65. Lee, J.H.; Lee, B.W.; Kim, B.; Kim, H.T.; Ko, J.M.; Baek, I.Y.; Seo, T.W.; Kang, M.Y.; Cho, K.M. Changes in phenolic compounds (isoflavones and phenolic acids) and antioxidant properties in high-protein soybean (*Glycine max* L., cv. Saedanbaek) for different roasting conditions. *J. Korean Soc. Appl. Biol. Chem.* **2013**, *56*, 605–612. [CrossRef]
66. United States Department of Agriculture (USDA). USDA Database for the Flavonoid Content of Selected Foods. 2013. Available online: https://www.ars.usda.gov/ARSUserFiles/80400525/Data/Flav/Flav3-1.pdf (accessed on 5 May 2017).
67. Baião, D.S.; Conte-Junior, C.A.; Paschoalin, V.M.F.; Alvares, T.S. Quantitative and comparative contents of nitrate and nitrite in *Beta vulgaris* L. by reversed-phase high-performance liquid chromatography-fluorescence. *Food Anal. Methods* **2016**, *9*, 1002–1008. [CrossRef]
68. Van Velzen, A.G.; Sips, A.J.; Schothorst, R.C.; Lambers, A.C.; Meulenbelt, J. The oral bioavailability of nitrate from nitrate-rich vegetables in humans. *Toxicol. Lett.* **2008**, *181*, 177–181. [CrossRef] [PubMed]
69. Váli, L.; Stefanovits-Bányai, E.; Szentmihályi, K.; Fébel, K.; Sárdi, E.; Lugasi, A.; Kocsis, I.; Blázovics, A. Liver-protecting effects of table beet (*Beta vulgaris* var. rubra) during ischemia-reperfusion. *Nutrition* **2007**, *23*, 172–178. [CrossRef] [PubMed]
70. Kanner, J.; Harel, S.; Granit, R. Betalains—A new class of dietary cationized antioxidants. *J. Agric. Food Chem.* **2001**, *49*, 5178–5185. [CrossRef] [PubMed]
71. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747. [PubMed]
72. Asami, D.K.; Hong, Y.J.; Barrett, D.M.; Mitchell, A.E. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. *J. Agric. Food Chem.* **2003**, *51*, 1237–1241. [CrossRef] [PubMed]
73. Čanadanović-Brunet, J.M.; Savatović, S.S.; Ćetković, G.S.; Vulić, J.J.; Djilas, S.M.; Markov, S.L.; Cvetković, D.D. Antioxidant and antimicrobial activities of beet root pomace extracts. *Czech J. Food Sci.* **2011**, *6*, 575–585.
74. Kavalcová, P.; Bystrická, J.; Tomáš, J.; Karoičová, J.; Kovarovič, J.; Lenková, M. The content of total polyphenols and antioxidante activity in red beetroot. *Potravinarstvo* **2015**, *9*, 77–83.
75. Wootton-Beard, P.C.; Ryan, L. A beetroot juice shot is a significant and convenient source of bioaccessible antioxidants. *J. Funct. Foods* **2011**, *3*, 329–334. [CrossRef]
76. Baião, D.S.; Conte-Junior, C.A.; Paschoalin, V.M.F.; Alvares, T.S. Beetroot juice increase nitric oxide metabolites in both men and women regardless of body mass. *Int. J. Food Sci. Nutr.* **2016**, *67*, 40–46. [CrossRef] [PubMed]
77. Vasconcellos, J.; Paschoalin, V.M.F.; Conte-Junior, C.A.; Alvares, T.S. Comparison of total antioxidant potential, and total phenolic, nitrate, sugar, and organic acid contents in beetroot juice, chips, powder, and cooked beetroot. *Food Sci. Biotechnol. (Seoul)* **2016**, *25*, 79–84. [CrossRef]
78. Da Silva, D.V.T.; Silva, F.O.; Moreira, D.P.; Pierucci, A.P.R.T.; Conte-Junior, C.A.; Silveira, T.A.; Del Aguila, E.M.; Paschoalin, V.M.F. Physicochemical, nutritional and sensory analyses of a nitrate-enriched beetroot gel and its effects on plasmatic nitric oxide and blood pressure. *Food Nutr. Res.* **2016**, *60*, 1–6. [CrossRef] [PubMed]
79. Baião, D.S.; da Silva, D.V.; Del Aguila, E.M.; Paschoalin, V.M.F. Nutritional, bioactive and physicochemical characteristics of different beetroot formulations. In *Food Additives*; InTech: Rijeka, Croatia, 2017; pp. 1–24.
80. Montagnac, J.A.; Davis, C.R.; Tanumihardjo, S.A. Nutritional value of cassava for use as a staple food and recent advances for improvement. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8*, 181–194. [CrossRef]
81. Pandey, A.; Soccol, C.R.; Nigam, P.; Soccol, V.T. Biotechnological potential of agro-industrial residues. II: Cassava bagasse. *Bioresour. Technol.* **2000**, *74*, 81–87. [CrossRef]

82. Food and Agriculture Organization (FAO). *A Review of Cassava in Latin America and the Caribbean with Country Case Studies on Brazil and Colombia*; FAO: Rome, Italy, 2004; Volume 4.
83. Alvarado, P.M.; Grosmaire, L.; Dufour, D.; Toro, A.G.; Sánchez, T.; Calle, F.; Santander, M.A.M.; Ceballos, H. Combined effect of fermentation, sun-drying and genotype on bread making ability of sour cassava starch. *Carbohydr. Polym.* **2013**, *98*, 1137–1146. [CrossRef] [PubMed]
84. Rebouças, K.H.; Gomes, L.P.; Leite, A.M.O.; Uekane, T.M.; Rezende, C.M.; Tavares, M.I.B.; Almeida, E.L.; Del Aguila, E.M.; Paschoalin, V.M.F. Evaluating physicochemical and rheological characteristics and microbial community dynamics during the natural fermentation of cassava starch. *J. Food Process. Technol.* **2016**, *7*, 4–11. [CrossRef]
85. Oladunmoye, O.O.; Aworh, O.C.; Maziya-Dixon, B.; Erukainure, O.L.; Elemo, G.N. Chemical and functional properties of cassava starch, durum wheat semolina flour, and their blends. *Food Sci. Nutr.* **2014**, *2*, 132–138. [CrossRef] [PubMed]
86. Dwivedi, S.C.; Dudey, R.; Richa, T.; Meeta, M.; Advani, U. Medicinal bioactives as antimicrobial agents: An overview. *Int. J. Pharm. Dev.* **2011**, *3*, 24–30.
87. Ferreres, F.; Gonçalves, R.F.; Gil-Izquierdo, A.; Valentão, P.; Silva, A.M.; Silva, J.B.; Santos, D.; Andrade, P.B. Further knowledge on the phenolic profile of *Colocasia esculenta* (L.) Shott. *J. Agric. Food Chem.* **2012**, *60*, 7005–7015. [CrossRef] [PubMed]
88. Bahekar, S.; Kale, R. Phytopharmacological aspects of *Manihot esculenta* Crantz (cassava)—A Review. *Mintage J. Pharm. Med. Sci.* **2013**, *2*, 4–5.
89. Adam, S.I.Y.; Ahmed, W.A.A.; Esra, O.M.; Nazik, E.Y.; Eiman, F.A.; Shaimaa, A.A.; Abdelgadir, W.S. Antimcrobial activity of Manihot esculenta root methanolic and aqueous extracts. *Eur. J. Biomed. Pharm. Sci.* **2014**, *1*, 390–401.
90. Sakai, T.; Nakagawa, Y. Diterpenic stress metabolites from cassava roots. *Phytochemistry* **1988**, *27*, 3769–3779. [CrossRef]
91. Uarrota, V.G.; Moresco, R.; Coelho, B.; da Nunes, E.C.; Peruch, L.A.; Neubert, O.; Rocha, M.; Maraschin, M. Metabolomics combined with chemometric tools (PCA, HLA, PLS-DA and SVM) for screening cassava (*Manihot esculenta*, Crantz) roots during postharvest physiological deterioration. *Food Chem.* **2014**, *161*, 67–78. [CrossRef] [PubMed]
92. Rickard, J.E. Biochemical changes involved in the postharvest deterioration of cassava roots. *Trop. Sci.* **1981**, *23*, 235–237.
93. Uarrota, V.G.; Nunes, E.C.; Peruch, L.A.M.; Neubert, E.O.; Coelho, B.; Moresco, R.; Ceballos, H. Toward better understanding of postharvest deterioration: Biochemical changes in stored cassava (*Manihot esculenta* Crantz) roots. *Food Sci. Nutr.* **2016**, *4*, 409–422. [CrossRef] [PubMed]
94. Oleszek, W.; Sitek, M.; Stochmal, A.; Piacente, S.; Pizza, C.; Cheeke, P. Resveratrol and other phenolics from the bark of Yucca schidigera Roezl. *J. Agric. Food Chem.* **2001**, *49*, 747–752. [CrossRef] [PubMed]
95. Piacente, S.; Montoro, P.; Oleszek, W.; Pizza, C. Yucca schidigera bark: Phenolic constituents and antioxidant activity. *J. Nat. Prod.* **2004**, *67*, 882–885. [CrossRef] [PubMed]
96. Fasuyi, A.O. Nutrient composition and processing effects on cassava leaf (*Manihot esculenta*, Crantz) antinutrients. *Pak. J. Nutr.* **2005**, *4*, 37–42.
97. Okeke, C.U.; Iweala, E. Antioxidant profile of *Dioscorea rotundata*, *Manihot esculenta*, *Ipoemea batatas*, *Vernonia amygdalina* and *Aloe vera*. *Med. Res. Technol.* **2007**, *4*, 4–10.
98. Bradbury, J.H.; Holloway, W.D. *Chemistry of Tropical Root Crops: Significance for Nutrition and Agriculture in Pacific*; ACIAR: Canberra, Australia, 1988.
99. Barthet, V.J. Polyphenol Oxidases from Cassava (*Manihot esculenta* C.) Root: Extraction, Purification and Characterization. Ph.D. Thesis, University McGill (Macdonald Campus), Montreal, QC, Canada, 1997; p. 179.
100. Chandrika, G.U.; Svanberg, U.; Jansz, R. In vitro accessibility of -carotene from cooked Sri Lankan green leafy vegetables and their estimated contribution to vitamin A requirement. *J. Sci. Food Agric.* **2006**, *86*, 54–61. [CrossRef]
101. Wobeto, C.; Corrêa, A.D.; Abreu, C.M.P.; Santos, C.D.; Abreu, J.R. Nutrients in the cassava (*Manihot esculenta* Crantz) leaf powder at three ages of the plant. *Food Sci. Technol.* **2006**, *26*, 865–869.
102. Reed, J.D.; McDowell, R.E.; Van Soest, P.J.; Horvath, P.J. Condensed tannins: A factor limiting the use of cassava forage. *J. Sci. Food. Agric.* **1982**, *33*, 213–220. [CrossRef]

103. Simão, A.A.; Santos, M.A.; Fraguas, R.M.; Braga, M.A.; Marques, T.R.; Duarte, M.H.; Santos, C.M.; Freire, J.M.; Correa, A.D. Antioxidants and chlorophyll in cassava leases at three plant ages. *Afr. J. Agric. Res.* **2013**, *8*, 3724–3730.
104. Almeida, S.E.M.; Arruda, S.F.; Vargas, R.M.; Souza, E.M.T. β-Carotene from cassava (*Manihot esculenta* Crantz) leaves improves vitamin A status in mice. *Compos. Biochem. Phys. C* **2007**, *146*, 235–240.
105. Buschmann, H.; Reilly, K.; Rodriguez, M.X.; Tohme, J.; Beeching, J.R. Hydrogen peroxide and flavan-3-ols in storage roots of cassava (*Manihot esculenta* Crantz) during postharvest deterioration. *J. Agric. Food Chem.* **2000**, *48*, 5522–5529. [CrossRef] [PubMed]
106. Simão, A.A.; Lage, F.F.; Chaga, P.M.B.; Fraguas, R.M.; Frei, J.M.; Marques, T.R.; Corrêa, A.D. Antioxidants from medicinal plants used in the treatment of obesity. *Eur. J. Med. Plants* **2013**, *3*, 429–443. [CrossRef]
107. Suresh, R.; Saravanakumar, M.; Suganyadevi, P. Anthocyanins from Indian cassava (*Manihot esculenta* Crantz) and its antioxidant properties. *Int. J. Pharm. Sci. Res.* **2011**, *37*, 1819–1828.
108. Tao, H.-T.; Qiu, B.; Du, F.-L.; Xu, T.-C.; Liu, L.-N.; Lu, F.; Li, K.-M.; Liu, W. The protective effects of cassava (*Manihot esculenta* Crantz) leaf flavonoid extracts on liver damage of carbon tetrachloride injured mice. *Afr. J. Tradit. Complement. Altern. Med.* **2015**, *12*, 52–56. [CrossRef]
109. Bokanisereme, U.F.Y.; Okechukwu, P.N. Anti-inflammatory, analgesic and anti-pyretic activity of cassava leaves extract. *Asian J. Pharm. Clin. Res.* **2013**, *6*, 89–92.
110. Montoro, P.; Skhirtladze, A.; Bassarello, C.; Perrone, A.; Kemertelidze, E.; Pizza, C.; Piacente, S. Determination of phenolic compounds in Yucca gloriosa bark and root by LC-MS/MS. *J. Pharm. Biomed. Anal.* **2008**, *47*, 854–859. [CrossRef] [PubMed]
111. Nair, K.P. *The Agronomy and Economy of Important Tree Crops of the Developing World*; Elsevier Inc.: Amsterdam, The Netherlands, 2010.
112. Jahurul, M.; Zaidul, I.; Norulaini, N.; Sahena, F.; Jinap, S.; Azmir, J.; Sharif, K.; Omar, A.M. Cocoa butter fats and possibilities of substitution in food products concerning cocoa varieties, alternative sources, extraction methods, composition, and characteristics. *J. Food Eng.* **2013**, *117*, 467–476. [CrossRef]
113. Do Carmo Brito, B.D.N.; Chisté, R.C.; da Silva Pena, R.; Gloria, M.B.A.; Lopes, A.S. Bioactive amines and phenolic compounds in cocoa beans are affected by fermentation. *Food Chem.* **2017**, *228*, 484–490. [CrossRef] [PubMed]
114. Minifie, B. *Chocolate, Cocoa and Confectionery: Science and Technology*; Springer Science & Business Media: New York, NY, USA, 2012; pp. 3–135.
115. Bordiga, M.; Locatelli, M.; Travaglia, F.; Coïsson, J.D.; Mazza, G.; Arlorio, M. Evaluation of the effect of processing on cocoa polyphenols: Antiradical activity, anthocyanins and procyanidins profiling from raw beans to chocolate. *Int. J. Food Sci. Technol.* **2015**, *50*, 840–848. [CrossRef]
116. Batista, N.N.; de Andrade, D.P.; Ramos, C.L.; Dias, D.R.; Schwan, R.F. Antioxidant capacity of cocoa beans and chocolate assessed by FTIR. *Food Res. Int.* **2016**, *90*, 313–319. [CrossRef]
117. D'Souza, R.N.; Grimbs, S.; Behrends, B.; Bernaert, H.; Ullrich, M.S.; Kuhnert, N. Origin-based polyphenolic fingerprinting of *Theobroma cacao* in unfermented and fermented beans. *Food Res. Int.* **2017**, *1*, 550–559. [CrossRef] [PubMed]
118. Camu, N.; De Winter, T.; Addo, S.K.; Takrama, J.S.; Bernaert, H.; De Vuyst, L. Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavour of chocolate. *J. Sci. Food Agric.* **2008**, *88*, 2288–2297. [CrossRef]
119. Paoletti, R.; Poli, A.; Conti, A.; Visioli, F. *Chocolate and Health*; Springer: Milan, Italy, 2012.
120. Ellam, S.; Williamson, G. Cocoa and human health. *Ann. Rev. Nutr.* **2013**, *33*, 105–128. [CrossRef] [PubMed]
121. Bastos, V.S. Sucessão Microbiana e Dinâmica de Substratos e Metabólitos Durante a Fermentação Espontânea de Grãos de Cacau (*Theobroma cacao* L.), Variedade Clonal 565, Cultivado no sul da Bahia. Ph.D. Thesis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, 2016.
122. De Vuyst, L.; Weckx, S. The cocoa bean fermentation process: From ecosystem analysis to starter culture development. *J. Appl. Microbiol.* **2016**, *121*, 5–17. [CrossRef] [PubMed]
123. Utami, R.R.; Armunanto, R.; Supriyanto, S.R.A. Effects of cocoa bean (*Theobroma cacao* L.) fermentation on phenolic content, antioxidant activity and functional group of cocoa bean shell. *Pak. J. Nutr.* **2016**, *15*, 948–953.
124. Nazaruddin, R.; Seng, L.; Hassan, O.; Said, M. Effect of pulp preconditioning on the content of polyphenols in cocoa beans (*Theobroma cacao*) during fermentation. *Ind. Crops Prod.* **2006**, *24*, 87–94. [CrossRef]

125. Sánchez-Rabaneda, F.; Jáuregui, O.; Casals, I.; Andrés-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventós, R.M. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J. Mass Spectrom.* **2003**, *38*, 35–42. [CrossRef] [PubMed]
126. Fernández-Murga, L.; Tarín, J.; García-Perez, M.; Cano, A. The impact of chocolate on cardiovascular health. *Maturitas* **2011**, *69*, 312–321. [CrossRef] [PubMed]
127. Hooper, L.; Kay, C.; Abdelhamid, A.; Kroon, P.A.; Cohn, J.S.; Rimm, E.B.; Cassidy, A. Effects of chocolate, cocoa, and flav-3-ols on cardiovascular health: A systematic review and meta-analysis of randomized trials. *Am. J. Clin. Nutr.* **2012**, *95*, 740–751. [CrossRef] [PubMed]
128. Kuebler, U.; Arpagaus, A.; Meister, R.E.; von Känel, R.; Huber, S.; Ehlert, U.; Wirtz, P.H. Dark chocolate attenuates intracellular pro-inflammatory reactivity to acute psychosocial stress in men: A randomized controlled trial. *Brain Behav. Immun.* **2016**, *57*, 200–208. [CrossRef] [PubMed]
129. Almoosawi, S.; Fyfe, L.; Ho, C.; Al-Dujaili, E. The effect of polyphenol-rich dark chocolate on fasting capillary whole blood glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects. *Br. J. Nutr.* **2010**, *103*, 842–850. [CrossRef] [PubMed]
130. Desideri, G.; Kwik-Uribe, C.; Grassi, D.; Necozione, S.; Ghiadoni, L.; Mastroiacovo, D.; Raffaele, A.; Ferri, L.; Bocale, R.; Lechiara, M.C. Benefits in cognitive function, blood pressure, and insulin resistance through cocoa flavanol consumption in elderly subjects with mild cognitive impairment novelty and significance. *Hypertension* **2012**, *60*, 794–801. [CrossRef] [PubMed]
131. FAO (Food and Agriculture Organization of the United Nations). 2017. Available online: http://www.fao.org/home/en/ (accessed on 19 July 2017).
132. Malenčić, D.; Maksimović, Z.; Popović, M.; Miladinović, J. Polyphenol contents and antioxidant activity of soybean seed extracts. *Bioresour. Technol.* **2008**, *99*, 6688–6691. [CrossRef] [PubMed]
133. Kuligowski, M.; Pawłowska, K.; Jasińska-Kuligowska, I.; Nowak, J. Isoflavone composition, polyphenols content and antioxidative activity of soybean seeds during tempeh fermentation. *CyTA-J. Food* **2017**, *15*, 27–33. [CrossRef]
134. Snyder, H.E.; Wilson, L.A. *Em Encyclopedia of Food Sciences and Nutrition*, 2nd ed.; Caballero, B., Trugo, L.C., Finglas, P.M., Eds.; Academic Press: Oxford, UK, 2003; Volume 9, pp. 5383–5389.
135. Aguiar, C.L.; Haddad, R.; Eberlin, M.N.; Carrao-Panizzi, M.C.; Mui, T.S.; Park, Y.K. Thermal behavior of malonylglucoside isoflavones in soybean flour analyzed by RPHPLC/DAD and eletrospray ionization mass spectrometry. *LWT Food Sci. Technol.* **2012**, *48*, 114–119. [CrossRef]
136. Tepavčević, V.; Atanacković, M.; Miladinović, J.; Malenčić, D.; Popović, J.; Cvejić, J. Isoflavone composition, total polyphenolic content, and antioxidant activity in soybeans of different origin. *J. Med. Food.* **2010**, *13*, 657–664. [CrossRef] [PubMed]
137. Barnes, S.; Kim, H.; Darley-Usmar, V.; Patel, R.; Xu, J.; Boersma, B.; Luo, M. Beyond ERα and ERβ: Estrogen receptor binding is only part of the isoflavone story. *J. Nutr.* **2000**, *130*, 656S–657S. [PubMed]
138. Heleno, S.A.; Martins, A.; Queiroz, M.J.R.; Ferreira, I.C. Bioactivity of phenolic acids: Metabolites versus parent compounds: A review. *Food Chem.* **2015**, *173*, 501–513. [CrossRef] [PubMed]
139. Andrade, C.M.; Sá, M.F.; Toloi, M.R. Effects of phytoestrogens derived from soy bean on expression of adhesion molecules on HUVEC. *Climacteric* **2012**, *15*, 186–194. [CrossRef] [PubMed]
140. Zakir, M.M.; Freitas, I.R. Benefícios à saúde humana do consumo de isoflavonas presentes em produtos derivados da soja. *J. Bioenergy Food Sci.* **2015**, *2*, 107–116. [CrossRef]
141. Marini, H.; Minutoli, L.; Polito, F.; Bitto, A.; Altavilla, D.; Atteritano, M.; Gaudio, A.; Mazzaferro, S.; Frisina, A.; Frisina, N.; et al. Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: A randomized trial. *Ann. Intern. Med.* **2007**, *146*, 839–847. [CrossRef] [PubMed]
142. Thomas, A.J.; Ismail, R.; Taylor-Swanson, L.; Cray, L.; Schnall, J.G.; Mitchell, E.S.; Woods, N.F. Effects of isoflavones and amino acid therapies for hot flashes and co-occurring symptoms during the menopausal transition and early postmenopause: A systematic review. *Maturitas* **2014**, *78*, 263–276. [CrossRef] [PubMed]
143. Uehara, M. Isoflavone metabolism and bone-sparing effects of daidzein-metabolites. *J. Clin. Biochem. Nutr.* **2013**, *52*, 193–201. [CrossRef] [PubMed]
144. Koh, K.; Youn, J.E.; Kim, H.S. Identification of anthocyanins in black soybean (*Glycine max* (L.) Merr.) varieties. *Food Sci. Technol.* **2014**, *51*, 377–381. [CrossRef] [PubMed]

145. Cho, K.M.; Ha, T.J.; Lee, Y.B.; Seo, W.D.; Kim, J.Y.; Ryu, H.W.; Lee, J.H. Soluble phenolics and antioxidant properties of soybean (*Glycine max* L.) cultivars with varying seed coat colours. *J. Funct. Foods* **2013**, *5*, 1065–1076. [CrossRef]
146. Prajapati, R.; Kalariya, M.; Umbarkar, R.; Parmar, S.; Sheth, N. *Colocasia esculenta*: A potent indigenous plant. *Int. J. Nutr. Pharmacol. Neurol. Dis.* **2011**, *1*, 90–96. [CrossRef]
147. Zárate, N.A.H.; Vieira, M.C.; Hiane, P.A. Produção e composição nutritiva de taro em função do propágulo, em solo hidromórfico do Pantanal Sul-Mato-Grossense. *Semin. Cien. Agrar.* **2006**, *27*, 361–366. [CrossRef]
148. Zárate, N.A.H.; Vieira, M.C.; Rego, N.H. Produtividade de clones de taro em função da população de plantas na época seca do pantanal sul-mato-grossense. *Pesqui. Agropecu. Trop.* **2007**, *36*, 141–143.
149. Oscarsson, K.V.; Savage, G.P. Composition and availability of soluble and insoluble oxalates in raw and cooked taro (*Colocasia esculenta* var. Schott) leaves. *Food Chem.* **2007**, *101*, 559–562. [CrossRef]
150. Owusu-Darko, P.G.; Paterson, A.; Omenyo, E.L. Cocoyam (corms and cormels)—An underexploited food and feed resource. *J. Agric. Chem. Environ.* **2014**, *3*, 22–29. [CrossRef]
151. Adegunwa, M.O.; Alamu, E.O.; Omitogun, L.A. Effect of processing on the nutritional contents of yam and cocoyam tubers. *J. Appl. Biosci.* **2011**, *6*, 3086–3092.
152. Olajide, R.; Akinsoyinu, A.O.; Babayemi, O.J.; Omojola, A.B.; Abu, A.O.; Afolabi, K.D. Effect of processing on energy values, nutrient and anti-nutrient components of wild cocoyam (*Colocasia esculenta* (L.) Schott) corm. *Pak. J. Nutr.* **2011**, *10*, 29–34. [CrossRef]
153. McDougall, G.J. Phenolic-enriched foods: Sources and processing for enhanced health benefits. *Proc. Nutr. Soc.* **2017**, *76*, 163–171. [CrossRef] [PubMed]
154. Gonçalves, R.F.; Silva, A.M.; Silva, A.M.; Valentão, P.; Ferreres, F.; Gil-Izquierdo, A.; Silva, J.B.; Santos, D.; Andrade, P.B. Influence of taro (*Colocasia esculenta* L. Shott) growth conditions on the phenolic composition and biological properties. *Food Chem.* **2013**, *141*, 3480–3485. [CrossRef] [PubMed]
155. Agyare, C.; Boakye, Y.D.; Apenteng, J.A.; Dapaah, S.O.; Appiah, T. Antimicrobial and anti-inflammatory properties of *Anchomanes difformis* (Bl.) Engl. and *Colocasia esculenta* (L.) Schott. *Biochem. Pharmacol.* **2016**, *5*, 201.
156. Simsek, S.; El, S.N. In vitro starch digestibility, estimated glycemic index and antioxidant potential of taro (*Colocasia esculenta* L. Schott) corm. *Food Chem.* **2015**, *168*, 257–261. [CrossRef] [PubMed]
157. Takebayashi, J.; Oki, T.; Watanabe, J.; Yamasaki, K.; Chen, J.; Sato-Furukawa, M.; Tsubota-Utsugi, M.; Taku, K.; Kazuhisa, G. Hydrophilic antioxidant capacities of vegetables and fruits commonly consumed in Japan and estimated average daily intake of hydrophilic antioxidants from these foods. *J. Food Compos. Anal.* **2013**, *29*, 25–31. [CrossRef]
158. Awa, E.; Eleazu, C. Bioactive constituents and antioxidant activities of raw and processed cocoyam (*Colocasia esculenta*). *Nutrafoods* **2015**, *14*, 133–140. [CrossRef]
159. Terasawa, N.; Saotome, A.; Tachimura, Y.; Mochizuki, A.; Ono, H.; Takenaka, M.; Murata, M. Identification and some properties of anthocyanin isolated from Zuiki, stalk of Colocasia esculenta. *J. Agric. Food Chem.* **2007**, *55*, 4154–4159. [CrossRef] [PubMed]
160. Alcantara, M.; Hurtada, A.; Dizon, I. The nutritional value and phytochemical components of taro *Colocasia esculenta* (L.) Schott powder and its selected processed foods. *J. Nutr. Food Sci.* **2013**, *3*, 3–10. [CrossRef]
161. Vauzour, D.; Rodriguez-Mateos, A.; Corona, G.; Oruna-Concha, M.J.; Spencer, J.P.E. Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients* **2010**, *2*, 1106–1131. [CrossRef] [PubMed]
162. Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81*, 243S–255S. [PubMed]
163. Rimbach, G.; Melchin, M.; Moehring, J.; Wagner, A.E. Polyphenols from Cocoa and Vascular Health—A Critical Review. *Int. J. Mol. Sci.* **2009**, *10*, 4290–4309. [CrossRef] [PubMed]
164. Potter, S.M.; Baum, J.A.; Teng, H.; Stillman, R.J.; Shay, N.F.; Erdman, J.W., Jr. Soy protein and isoflavones: Their effects on blood lipids and bone density in postmenopausal women. *Am. J. Clin. Nutr.* **1998**, *68*, 1375S–1379S. [PubMed]
165. Morabito, N.; Crisafulli, A.; Vergara, C.; Gaudio, A.; Lasco, A.; Frisina, N.; D'Anna, R.; Corrado, F.; Pizzoleo, M.A.; Cincotta, M.; et al. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: A randomized double-blind placebo-controlled study. *J. Bone Miner. Res.* **2002**, *17*, 1904–1912. [CrossRef] [PubMed]

166. Wiseman, H.; O'Reilly, J.D.; Adlercreutz, H.; Mallet, A.I.; Bowey, E.A.; Rowland, I.R.; Sanders, T.A. Isoflavone phytoestrogens consumed in soy decrease F(2)-isoprostane concentrations and increase resistance of low-density lipoprotein to oxidation in humans. *Am. J. Clin. Nutr.* **2000**, *72*, 395–400. [PubMed]
167. Mathur, S.; Devaraj, S.; Grundy, S.M.; Jialal, I. Cocoa products decrease low density lipoprotein oxidative susceptibility but do not affect biomarkers of inflammation in humans. *J. Nutr.* **2002**, *132*, 3663–3667. [PubMed]
168. Fisher, N.D.; Hughes, M.; Gerhard-Herman, M.; Hollenberg, N.K. Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *J. Hypertens.* **2003**, *21*, 2281–2286. [CrossRef] [PubMed]
169. Heiss, C.; Dejam, A.; Kleinbongard, P.; Schewe, T.; Sies, H.; Kelm, M. Vascular effects of cocoa rich in flavan-3-ols. *JAMA* **2003**, *290*, 1030–1031. [CrossRef] [PubMed]
170. Flammer, A.J.; Hermann, F.; Sudano, I.; Spieker, L.; Hermann, M.; Cooper, K.A.; Serafini, M.; Luscher, T.F.; Ruschitzka, F.; Noll, G.; et al. Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation* **2007**, *116*, 2376–2382. [CrossRef] [PubMed]
171. Balzer, J.; Rassaf, T.; Heiss, C.; Kleinbongard, P.; Lauer, T.; Merx, M.; Heussen, N.; Gross, H.B.; Keen, C.L.; Schroeter, H.; et al. Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial. *J. Am. Coll. Cardiol.* **2008**, *51*, 2141–2149. [CrossRef] [PubMed]
172. Clifford, T.; Howatson, G.; West, D.J.; Stevenson, E.J. The potential benefits of red beetroot supplementation in health and disease. *Nutrients* **2015**, *7*, 2801–2822. [CrossRef] [PubMed]
173. Luqman, S.; Rizvi, S.I. Protection of lipid peroxidation and carbonyl formation in proteins by capsaicin in human erythrocytes subjected to oxidative stress. *Phytother. Res.* **2006**, *20*, 303–306. [CrossRef] [PubMed]
174. Andújar, I.; Recio, M.C.; Giner, R.M.; Rios, J.L. Cocoa polyphenols and their potential benefits for human health. *Oxid. Med. Cell. Longev.* **2012**, *2012*, 906252. [CrossRef] [PubMed]
175. García-Lafuente, A.; Guillamón, E.; Villares, A.; Rostagno, M.A.; Martínez, J.A. Flavonoids as antiinflammatory agents: Implications in cancer and cardiovascular disease. *Inflamm. Res.* **2009**, *58*, 537–552. [CrossRef] [PubMed]
176. Balestrieri, C.; Felice, F.; Piacente, S.; Pizza, C.; Montoro, P.; Oleszek, W.; Visciano, V.; Balestrieri, M.L. Relative effects of phenolic constituents from *Yucca schidigera* Roezl. bark on Kaposi's sarcoma cell proliferation, migration, and PAF synthesis. *Biochem. Pharmacol.* **2006**, *71*, 1479–1487. [CrossRef] [PubMed]
177. Yamori, Y.; Moriguchi, E.H.; Teramoto, T.; Miura, A.; Fukui, Y.; Honda, K.; Fukui, M.; Nara, Y.; Taira, K.; Moriguchi, Y. Soybean isoflavones reduce postmenopausal bone resorption in female Japanese immigrants in Brazil: A ten-week study. *J. Am. Coll. Nutr.* **2002**, *21*, 560–563. [CrossRef] [PubMed]
178. Yang, C.S.; Landau, J.M.; Huang, M.T.; Newmark, H.L. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Ann. Rev. Nutr.* **2001**, *21*, 381–406. [CrossRef] [PubMed]
179. Johnson, I.T.; Williamson, G.; Musk, S.R.R. Anticarcinogenic factors in plant foods: A new class of nutrients? *Nutr. Res. Rev.* **1994**, *7*, 175–204. [CrossRef] [PubMed]
180. Jenkins, D.J.; Kendall, C.W.; Garsetti, M.; Rosenberg-Zand, R.S.; Jackson, C.J.; Agarwal, S.; Rao, A.V.; Diamandis, E.P.; Parker, T.; Faulkner, D.; et al. Effect of soy protein foods on low-density lipoprotein oxidation and ex vivo sex hormone receptor activity—A controlled crossover trial. *Metabolism* **2000**, *49*, 537–543. [CrossRef]
181. Russo, J.; Russo, I.H. The role of estrogen in the initiation of breast cancer. *J. Steroid. Biochem. Mol. Biol.* **2006**, *102*, 89–96. [CrossRef] [PubMed]
182. Sung, H.; Nah, J.; Chun, S.; Park, H.; Yang, S.E.; Min, W.K. In vivo antioxidant effect of green tea. *Eur. J. Clin. Nutr.* **2000**, *54*, 527–529. [CrossRef] [PubMed]
183. Young, J.F.; Dragstedt, L.O.; Haraldsdottir, J.; Daneshvar, B.; Kall, M.A.; Loft, S.; Nilsson, L.; Nielsen, S.E.; Mayer, B.; Skibsted, L.H.; et al. Green tea extract only affects markers of oxidative status postprandially: Lasting antioxidant effect of flavonoid-free diet. *Br. J. Nutr.* **2002**, *87*, 343–355. [CrossRef] [PubMed]
184. Buijsse, B.; Feskens, E.J.; Kok, F.J.; Kromhout, D. Cocoa intake, blood pressure, and cardiovascular mortality: The Zutphen Elderly Study. *Arch. Intern. Med.* **2006**, *166*, 411–417. [CrossRef] [PubMed]
185. Kris-Etherton, P.M.; Keen, C.L. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Curr. Opin. Lipidol.* **2002**, *13*, 41–49. [CrossRef] [PubMed]

186. Hooper, L.; Kroon, P.A.; Rimm, E.B.; Cohn, J.S.; Harvey, I.; le Cornu, K.A.; Ryder, J.J.; Hall, W.L.; Cassidy, A. Flavonoids, flavonoid-rich foods, and cardiovascular risk: A meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **2008**, *88*, 38–50. [PubMed]
187. Jia, L.; Liu, X.; Bai, Y.Y.; Li, S.H.; Sun, K.; He, C.; Hui, R. Short-term effect of cocoa product consumption on lipid profile: A meta-analysis of randomized controlled trials. *Am. J. Clin. Nutr.* **2010**, *92*, 218–225. [CrossRef] [PubMed]
188. Ludovici, V.; Barthelmes, J.; Nägele, M.P.; Enseleit, F.; Ferri, C.; Flammer, A.J.; Ruschitzka, F.; Sudano, I. Cocoa, blood pressure, and vascular function. *Front. Nutr.* **2017**, *4*, 1–12. [CrossRef] [PubMed]
189. Baba, S.; Natsume, M.; Yasuda, A.; Nakamura, Y.; Tamura, T.; Osakabe, N.; Kanegae, M.; Kondo, K. Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. *J. Nutr.* **2007**, *137*, 1436–1441. [PubMed]
190. Di Renzo, L.; Rizzo, M.; Sarlo, F.; Colica, C.; Iacopino, L.; Domino, E.; Sergi, D.; De Lorenzo, A. Effects of dark chocolate in a population of normal weight obese women: A pilot study. *Eur. Rev. Med. Pharmacol. Sci.* **2013**, *17*, 2257–2266. [PubMed]
191. Monahan, K.D. Effect of cocoa/chocolate ingestion on brachial artery flow-mediated dilation and its relevance to cardiovascular health and disease in humans. *Arch. Biochem. Biophys.* **2012**, *527*, 90–94. [CrossRef] [PubMed]
192. Sies, H.; Schewe, T.; Heiss, C.; Kelm, M. Cocoa polyphenols and inflammatory mediators. *Am. J. Clin. Nutr.* **2005**, *81*, 304S–312S. [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Dietary Inflammatory Index and Colorectal Cancer Risk—A Meta-Analysis

Nitin Shivappa [1,2,3,*], Justyna Godos [4], James R. Hébert [1,2,3], Michael D. Wirth [1,2,3], Gabriele Piuri [4], Attilio F. Speciani [4] and Giuseppe Grosso [5,6]

1 Cancer Prevention and Control Program, University of South Carolina, Columbia, SC 29208, USA; JHEBERT@mailbox.sc.edu (J.R.H.); wirthm@mailbox.sc.edu (M.D.W.)
2 Department of Epidemiology and Biostatistics, Arnold School of Public Health, University of South Carolina, Columbia, SC 29208, USA
3 Connecting Health Innovations LLC, Columbia, SC 29201, USA
4 Inflammation Society, Church Hill, Orpington, London BR6OHH, UK; justynagodos@gmail.com (J.G.); gabriele.piuri@studiospeciani.it (G.P.); attilio.speciani@me.com (A.F.S.)
5 Integrated Cancer Registry of Catania-Messina-Siracusa-Enna, Azienda Ospedaliera Universitaria Policlinico Vittorio Emanuele, 95123 Catania, Italy; giuseppe.grosso@studium.unict.it
6 NNEdPro Global Centre for Nutrition and Health, St John's Innovation Centre, Cambridge CB4 0WS, UK
* Correspondence: shivappa@mailbox.sc.edu

Received: 8 August 2017; Accepted: 18 September 2017; Published: 20 September 2017

Abstract: Diet and chronic inflammation of the colon have been suggested to be risk factors in the development of colorectal cancer (CRC). The possible link between inflammatory potential of diet, measured through the Dietary Inflammatory Index (DII®), and CRC has been investigated in several populations across the world. The aim of this study was to conduct a meta-analysis on studies exploring this association. Data from nine studies were eligible, of which five were case-control and four were cohort studies. Results from meta-analysis showed a positive association between increasing DII scores, indicating a pro-inflammatory diet, and CRC. Individuals in the highest versus the lowest (reference) DII category showed an overall 40% increased risk of CRC with moderate evidence of heterogeneity [relative risk (RR) = 1.40, 95% confidence interval (CI): 1.26, 1.55; I^2 = 69%, $p < 0.001$]. When analyzed as a continuous variable, results showed an increased risk of CRC of 7% for a 1-point increase in the DII score. Results remained unchanged when analyses were restricted to the four prospective studies. Results of our meta-analysis support the importance of adopting a healthier anti-inflammatory diet in preventing CRC. These results further substantiate the utility of DII as tool to characterize the inflammatory potential of diet and to predict CRC.

Keywords: diet; cytokines; nutrition; inflammation; epidemiology; dietary inflammatory index; colorectal cancer; meta-analysis

1. Introduction

Colorectal cancer (CRC) is the third most common form of cancer worldwide and is one of the leading causes of cancer-related deaths [1]. Incidence and mortality rates of CRC vary widely with higher incidence rates in developed nations and lower incidence rates in Asia, Africa, and most Latin American countries [2]. Inflammation typically occurs as part of the body's normal response to tissue insult/injury [3,4]. Chronic inflammation is a persistent condition in which tissue destruction and repair occur simultaneously [5,6], involving continuous recruitment of pro-inflammatory cytokines (associated with increased blood flow to the injured tissue, due to histamine released by damaged mast cells) [3]. Increased levels of these cytokines also are believed to be associated with CRC [7–9]. Furthermore, some research suggests a direct association between specific dietary components and

inflammation [10–13]. Various dietary components may be involved in the development of CRC [14]. The 2012 American Institute for Cancer Research/World Cancer Research Fund Continuous Update Project (CUP) reported that consumption of red and processed meat, which are pro-inflammatory, is associated with an increased risk of CRC [14]. Conversely, the consumption of dietary fiber, which is anti-inflammatory, is inversely associated with risk of CRC [14]. Furthermore, other dietary components, such as tea and coffee, which we have found to be anti-inflammatory, have demonstrated various health benefits , including lower cancer incidence [15,16] and mortality [17,18]. Moreover, comprehensive investigations on whole dietary patterns have indicated that unhealthy dietary patterns are associated with higher risk of CRC and adenoma, while healthy diets are associated with lower risk [19,20].

In response to the absence of an instrument that could summarize diets' ability to influence inflammatory processes, in 2009 researchers at the University of South Carolina developed the first Dietary Inflammatory Index, which was created based on literature published on diet and inflammation through 2007 [21]. In 2014 the new refined and improved Dietary Inflammatory Index (DII®) was based on literature published on diet and inflammation through 2010 [22]. The DII categorizes individuals' diets according to their inflammatory potential on a continuum from maximally pro-inflammatory to maximally anti-inflammatory. A higher DII score indicates a more pro-inflammatory diet, whereas a lower DII score represents a more anti-inflammatory diet. The DII is composed of 45 food parameters, out of which 36 are anti-inflammatory. These include: fiber, alcohol, monounsaturated fatty acids, polyunsaturated fatty acids, omega 3, omega 6, niacin, thiamin, riboflavin, vitamin B6, B12, zinc, magnesium, selenium, vitamin A, vitamin C, vitamin D, vitamin E, folic acid, beta carotene, anthocyanidins, flavan3ols, flavonols, flavanones, flavones, isoflavones, garlic, ginger, onions, thyme, oregano, saffron, turmeric, rosemary, eugenol, caffeine, and tea. The remaining 9 are pro-inflammatory components: energy, carbohydrates, proteins, total fat, trans fat, cholesterol, vitamin B12, saturated fatty acids and iron. As a rule, foods that have low DII scores tend to be flavorful, colorful, nutrient-dense, and calorie-sparse. By contrast, those foods that have high DII scores tend to be flavorless (even though they may have a strong taste, such as sweet), are white or colorless, nutrient-sparse and calorie-dense. The DII was found to predict changes in high sensitivity-C-reactive protein (hs-CRP) in the Seasonal Variation in Blood Cholesterol Study [21,23]. Subsequently, the DII has been used in several studies from around the world to test the effect of diet-associated inflammation on inflammation markers such as CRP, interleukin (IL)-6, and (tumor necrosis factor) and TNF-α-R2 [23–30]. In the Seasonal Variation of Blood Cholesterol Study, higher DII scores were associated with values of hs-CRP > 3 mg/L [odds ratio (OR) = 1.08; 95% confidence interval (CI): 1.01, 1.16, $p = 0.035$ for the 24 hour recall (24 HR) subset; and OR = 1.10; 95% CI: 1.02, 1.19, $p = 0.015$ for the 7-Day Dietary Recall] [23]; in the Women's Health Initiative, the DII was associated with the four biomarkers with beta estimates comparing the highest with lowest DII quintiles as follows: Interleukin-6: 1.26 (1.15–1.38), $p_{\text{trend}} < 0.0001$; tumor necrosis factor alpha receptor 2: 81.43 (19.15–143.71), $p_{\text{trend}} = 0.004$; dichotomized hs-CRP (odds ratio for higher vs. lower hs-CRP): 1.30 (0.97–1.67), $p_{\text{trend}} = 0.34$; and the combined inflammatory biomarker score: 0.26 (0.12–0.40), $p_{\text{trend}} = 0.0001$ [24]. Additionally, the DII has been linked to various health outcomes including cancer incidence [31–33]; all-cause, cardiovascular and cancer-specific mortality [34–36]; respiratory conditions such as asthma [28,37]; and cognitive disorders [38,39]. The most consistent results have been observed with CRC, with nine studies published exploring this association [40–48]. The current meta-analysis aimed to investigate the cumulative association between the inflammatory potential of diet, as estimated by the DII score, and CRC risk based on the results from nine previous studies.

2. Methods

2.1. Search Strategy and Study Selection

Literature databases including PubMed, SCOPUS, and EMBASE were searched from beginning through July 2017. Relevant keywords related to the DII were searched in combination with keywords related to CRC {[(dietary inflammatory index OR inflammatory diet OR anti-inflammatory diet OR dietary score) AND (colorectal OR colon OR rectal OR rectum)] AND (cancer OR carcinoma OR neoplasm)}. Reference lists of retrieved articles were manually searched by two researchers (G.G. and S.N.). The literature search was limited to English. If more than one article was published using the same cohort, the most recent article with the longest follow-up period was considered. Studies included in this systematic review met all of the following inclusion criteria: (i) focused on humans and had a case-control or a prospective study design; and (ii) evaluated the risk or association between the DII and CRC. The two investigators independently assessed articles for compliance with the inclusion and exclusion criteria and resolved disagreements through consensus.

2.2. Data Extraction

The following information was extracted from each study: (i) name of the first author; (ii) year of publication; (iii) study cohort or name; (iv) country; (v) number of participants; (vi) sex of participants; (vii) age range or mean age of the study population at baseline; (viii) follow-up period; (ix) endpoints and cases; (x) measures of risk [hazard ratios (HRs)] or association [odds ratios (ORs)] with 95% confidence intervals (CIs) for the highest versus the lowest category of exposure and for 1-point increase of the DII score (when available); and (xi) covariates used for adjustment.

The quality of observational studies was assessed according to the Newcastle-Ottawa Quality Assessment Scale [49], consisting of three parameters of quality: selection (four points), comparability (two points), and outcome (three points), with a score of seven or more points reflecting high quality.

2.3. Statistical Analysis

In this meta-analysis, ORs and HRs were deemed equivalent to relative risks (RRs) [50]. Random- and fixed-effects models were used to calculate pooled RRs with 95% CIs of colorectal cancer for the highest compared to the lowest category of exposure and for a 1-point increase of the DII score. Risk estimates of CRC for 1-point increase of the score (continuous) also were estimated in studies not reporting the measure, but providing sufficient data to estimate it. Heterogeneity was assessed by using the Q test and I^2 statistic. The significance of the Q test was defined as $p < 0.10$. The I^2 statistic represents the amount of total variation that could be attributed to heterogeneity. I^2 values $\leq$25%, $\leq$50%, $\leq$75% and >75% indicated no, little, moderate, and high heterogeneity, respectively. A sensitivity analysis was conducted by excluding one study at a time in order to assess the stability of results. Subgroup analyses were conducted by tumor localization (colon, rectum), sex, geographical region [North America (n = 5), Europe (n = 2)], and adjustment for smoking, BMI, physical activity and non-steroidal anti-inflammatory drug (NSAID) use. Publication bias was assessed by visual observation of funnel plots. All analyses were performed with Review Manager (RevMan) version 5.2 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark).

3. Results

The relevance of studies was assessed with a hierarchical approach on the basis of title, abstract, and the full manuscript. The full process of identification and selection of studies is shown in Figure 1. The search strategy identified 1,003 studies, of which 925 were excluded after review of title, and 66 on the basis of abstract (Figure 1). Of the 12 publications selected, 3 were not included for the following reasons: (1) the article evaluated the association between different dietary score and CRC; (2) the study was a systematic review (and therefore did not present any new finding).

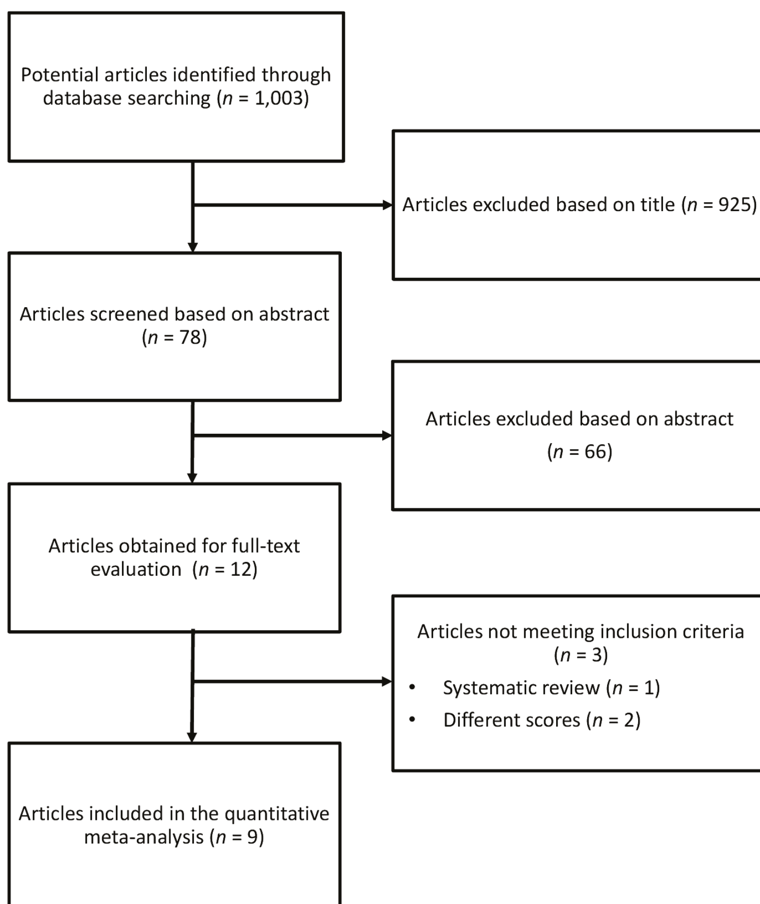


Figure 1. Flow chart and process selection of relevant studies exploring the association between Dietary Inflammatory Index (DII) and risk of colorectal, colon and rectal cancer.

The nine studies selected included a total of 881,612 individuals and 18,888 cases of colorectal cancer available for the present meta-analysis.

Table 1 shows the information extracted from all nine studies included. Five studies had a case-control design [40,44,46–48], which comprised 4000 cases and 7288 controls. Four studies were prospective cohorts [41–43,45], which comprised 715,088 participants and 14,888 incident cases of colorectal cancer; cohorts included the Iowa Women's Health Study (IWHS), the Women's Health Initiative (WHI), The National Institutes of Health–American Association of Retired Persons (NIH-AARP) Diet and Health Study, and the Multiethnic Cohort (MEC). All the studies included covariates that may have significant influence on colorectal cancer, such as age, sex (when not analyzed separately), BMI, education, physical activity, and smoking status. The comprehensive group of covariates used for adjustments are described in Table 1.

Table 1. Characteristics of studies included in the meta-analysis.

Author, Year	Study Design	Study Cohort, Country	Sex, Age Range/Mean (Years)	No. of Individuals/Controls	No. of Cases	Follow-Up (Years)	No. of Food Parameters to Calculate DII	Adjustments
Shivappa et al. 2014 [45]	Cohort	Iowa Women's Health Study, USA	Females, 62 ± 4	34,703	1636	19.6	37	Age, BMI, smoking status, pack-years of smoking, education, hormone replacement therapy use, total energy intake, NSAIDs and history of diabetes.
Wirth et al. 2015 [42]	Cohort	NIH-AARP, USA	Both males and females. Age: 62 ± 5.4	489,422	6944	9.1	35	Age, smoking status, BMI, self-reported diabetes, energy intake, physical activity, marital status, education, race and census-based income.
Harmon et al. 2017 [41]	Cohort	Multiethnic Cohort	Both males and females. Age: 45–75	190,963	4388	20	28	Age, sex, BMI, race, self-reported previous diagnosis of diabetes, asthma, and heart attack; use of supplements; smoking status; family history of colon cancer; education; hormone (i.e., estrogen or progesterone) use; aspirin use.
Tabung et al. 2015 [43]	Cohort	Women's Health Initiative, USA	Females. Age: 50–79	152,536	1920	11.3	32	Age, total energy intake, body mass index, race/ethnicity, physical activity, educational level, smoking status, family history of colorectal cancer, hypertension, diabetes, arthritis, history of colonoscopy, history of occult blood tests, NSAID use, category and duration of estrogen use, category and duration of estrogen & progesterone use, dietary modification trial arm, hormone therapy trial arm and calcium and vitamin trial arm
Shivappa et al. 2015 [48]	Case-control	Italy	Both males and females. Age: Case-60 ± 10 Controls-56 ± 11	4154 controls	1953	-	31	Age, sex, study center, education, BMI, alcohol consumption, physical activity, history of colorectal cancer, and energy intake
Zamora-Ros et al. 2015 [44]	Case-control	Spain	Both males and females. Age: 65.8 ± 12	401 controls	424	-	33	Age, sex, total energy intake, BMI, first-degree family history of CRC, physical activity, tobacco use, and medication use (aspirin and NSAID)
Cho et al. 2016 [46]	Case-control	South Korea	Both males and females. Age: Cases = 56.6 Control = 56.1	1846 controls	923	-	36	Age, sex, BMI, education, family history of colorectal cancer, physical activity, and total energy intake.
Shivappa et al. 2017 [40]	Case-control	Jordan	Both males and females. Age: Cases: 52 ± 11 Controls: 54 ± 12	202 controls	153	-	18	Age, sex, education, physical activity, body mass index, smoking, and family history of colorectal cancer.
Sharma et al. 2017 [47]	Case-control	Canada	Both males and females. Age: Cases: 62 ± 9 Controls: 60 ± 9	685 controls	547	-	29	Age, sex, BMI, physical activity, cholesterol level, triglycerides, family history of CRC, polyps, diabetes, history of colon screening, smoking, alcohol consumption, regular use of NSAIDs, and reported HRT, females only.

Individuals in the highest versus the lowest (reference) DII category of exposure had an overall 40% increased risk of colorectal cancer with moderate evidence of heterogeneity (RR = 1.40, 95% CI: 1.26, 1.55; I^2 = 69%, $p < 0.001$; Figure 2). Funnel plot results indicate that case-control studies generally reported higher risk estimates (Figure 3A). Both heterogeneity and funnel plot results (used to evaluate risk of publication bias) were driven by case-control studies (Figures 2 and 3B). However, analysis restricted to prospective cohorts alone showed essentially unchanged risk estimates, with only minor evidence of heterogeneity and no evidence of publication bias (RR = 1.24, 95% CI: 1.15, 1.35; I^2 = 50%, p = 0.08; Figures 2 and 3C). When using a fixed-effect model, risk estimates were essentially the same for colorectal cancer (RR = 1.32, 95% CI: 1.26, 1.39; I^2 = 69%, $p < 0.001$), and in separate analysis for prospective cohorts (RR = 1.26, 95% CI: 1.19, 1.33; I^2 = 50%, p = 0.08) and case-control studies (RR = 1.68, 95% CI: 1.49, 1.90; I^2 = 42%, p = 0.11). Subgroup analyses showed no differences between any of the groups investigated (Table 2).

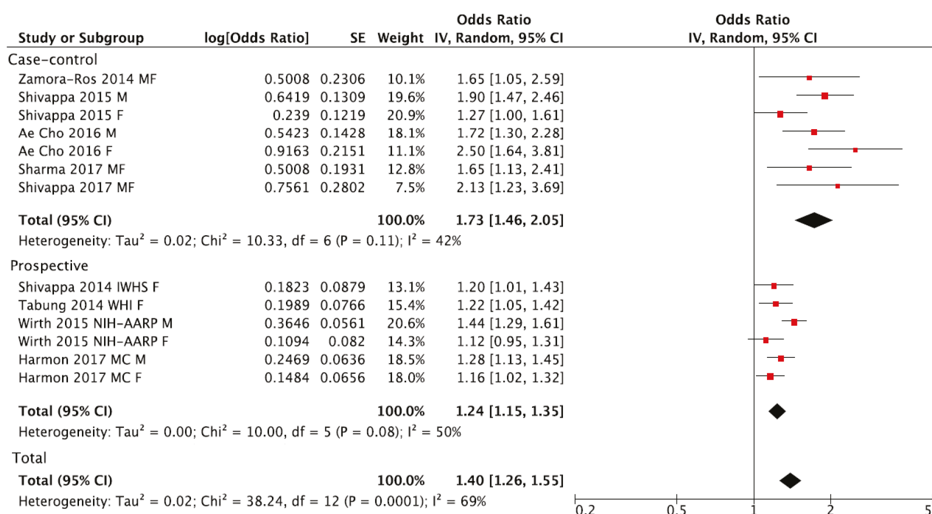


Figure 2. Forest plot of summary relative risks (RRs) of colorectal cancer for the highest versus lowest (reference) category of Dietary Inflammatory Index (DII), for case-control, prospective and all studies.

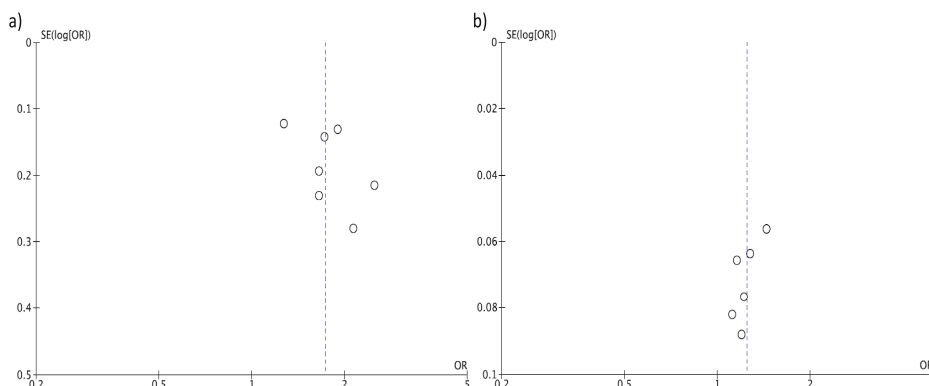


Figure 3. *Cont.*

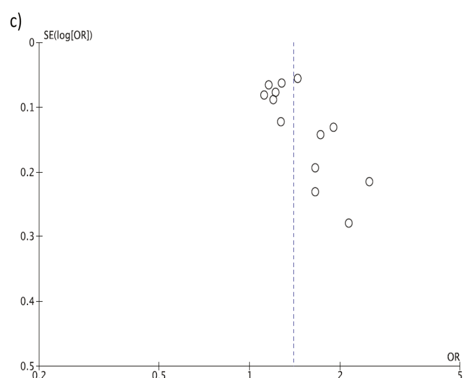


Figure 3. Funnel plots for colorectal cancer risk of the highest versus lowest (reference) category of Dietary Inflammatory Index (DII): (**a**) case-control, (**b**) prospective, and (**c**) all studies.

Table 2. Subgroup analyses of studies reporting risk of colorectal, colon and rectal cancer for the highest *versus* lowest (reference) category of dietary inflammatory index (DII).

Subgroup	No. of Datasets (No. of Studies)	RR (95% CI)	I^2 (%)	$P_{heterogeneity}$
Colorectal				
Total	13 (9)	1.40 (1.26, 1.55)	69%	0.0001
Study design				
Prospective	6 (4)	1.24 (1.15, 1.35)	50%	0.08
Case-control	7 (5)	1.73 (1.46, 2.05)	42%	0.11
Gender				
Men	4 (4)	1.51 (1.29, 1.75)	68%	0.02
Women	6 (6)	1.25 (1.10, 1.41)	61%	0.02
Geographical location				
North America	7 (5)	1.26 (1.16, 1.36)	50%	0.06
Europe	3 (2)	1.57 (1.19, 2.07)	61%	0.08
Asia	3 (2)	1.97 (1.57, 2.49)	9%	0.33
Adjustment for smoking				
No	4 (2)	1.74 (1.34, 2.25)	69%	0.02
Yes	9 (7)	1.28 (1.18, 1.40)	52%	0.03
Adjustment for BMI				
No	1 (1)	1.65 (1.13, 2.42)	NA	NA
Yes	12 (8)	1.39 (1.25, 1.54)	70%	0.0001
Adjustment for physical activity				
No	3 (2)	1.22 (1.12, 1.32)	0%	0.55
Yes	10 (7)	1.51 (1.31, 1.74)	70%	0.0004
Adjustment for NSAID				
No	8 (5)	1.50 (1.28, 1.75)	76%	0.0002
Yes	5 (4)	1.25 (1.15, 1.37)	21%	0.28
Colon				
Total	10 (7)	1.38 (1.23, 1.55)	61%	0.006
Study design				
Prospective	5 (4)	1.25 (1.16, 1.35)	11%	0.34
Case-control	5 (3)	1.70 (1.29, 2.24)	62%	0.03
Gender				
Men	3 (3)	1.58 (1.36, 1.83)	0%	0.71
Women	5 (5)	1.27 (1.10, 1.48)	51%	0.09
Geographical location				
North America	5 (4)	1.25 (1.16, 1.35)	11%	0.34
Europe	3 (2)	1.56 (1.06, 2.29)	71%	0.03
Asia	2 (1)	1.97 (1.34, 2.90)	39%	0.20

Table 2. *Cont.*

Subgroup	No. of Datasets (No. of Studies)	RR (95% CI)	I^2 (%)	$P_{heterogeneity}$
Adjustment for smoking				
No	4 (2)	1.62 (1.20, 2.19)	66%	0.03
Yes	6 (5)	1.29 (1.16, 1.43)	46%	0.10
Adjustment for BMI				
No	0 (0)	NA	NA	NA
Yes	10 (7)	1.38 (1.23, 1.55)	61%	0.006
Adjustment for physical activity				
No	2 (2)	1.20 (1.10, 1.31)	0%	0.94
Yes	8 (5)	1.48 (1.27, 1.72)	59%	0.02
Adjustment for NSAID				
No	7 (4)	1.43 (1.23, 1.66)	58%	0.03
Yes	3 (3)	1.29 (1.07, 1.56)	62%	0.07
Rectal				
Total	10 (7)	1.35 (1.18, 1.56)	48%	0.04
Study design				
Prospective	5 (4)	1.23 (1.03, 1.47)	54%	0.07
Case-control	5 (3)	1.55 (1.30, 1.85)	7%	0.36
Gender				
Men	3 (3)	1.56 (1.35, 1.81)	0%	0.75
Women	5 (5)	1.28 (0.97, 1.69)	59%	0.05
Geographical location				
North America	5 (4)	1.23 (1.03, 1.47)	54%	0.07
Europe	3 (2)	1.41 (1.15, 1.73)	0%	0.72
Asia	2 (1)	1.90 (1.41, 2.56)	3%	0.31
Adjustment for smoking				
No	4 (2)	1.60 (1.34, 1.91)	4%	0.37
Yes	6 (5)	1.22 (1.04, 1.44)	43%	0.12
Adjustment for BMI				
No	0 (0)	NA	NA	NA
Yes	10 (7)	1.35 (1.18, 1.56)	48%	0.04
Adjustment for physical activity				
No	2 (2)	1.22 (1.03, 1.43)	0%	0.97
Yes	8 (5)	1.40 (1.17, 1.68)	54%	0.03
Adjustment for NSAID				
No	7 (4)	1.43 (1.18, 1.73)	58%	0.03
Yes	3 (3)	1.21 (1.04, 1.41)	0%	0.96

The analysis considering the DII score as a continuous variable showed an increased risk of colorectal cancer of 7% for each 1-point increase of the score, despite the analysis being affected by similar limitations as the previous studies, such as high heterogeneity (Figure 4) and evidence of publication bias based on the funnel plot (Figure 5A). When considering only prospective studies, the association between a 1-point increase of the DII score and risk of colorectal cancer was significant, yet with moderate heterogeneity between results (RR = 1.03, 95% CI: 1.02, 1.04; I^2 = 58%, p = 0.03; Figure 4) but no evidence of publication bias at funnel plot (Figure 5C).

The analyses of separate datasets by tumor location showed similar risk of both colon and rectal cancer for the highest versus the lowest (reference) DII category of exposure, with similar characteristics reported for the general analysis (Figures 6 and 7).

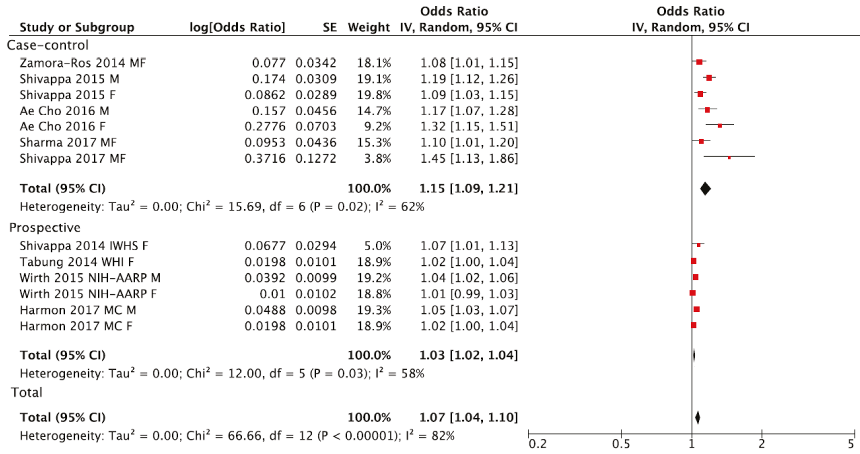


Figure 4. Forest plot of summary relative risks (RRs) of colorectal cancer for a one-point increase of Dietary Inflammatory Index (DII), for case-control, prospective and all studies.

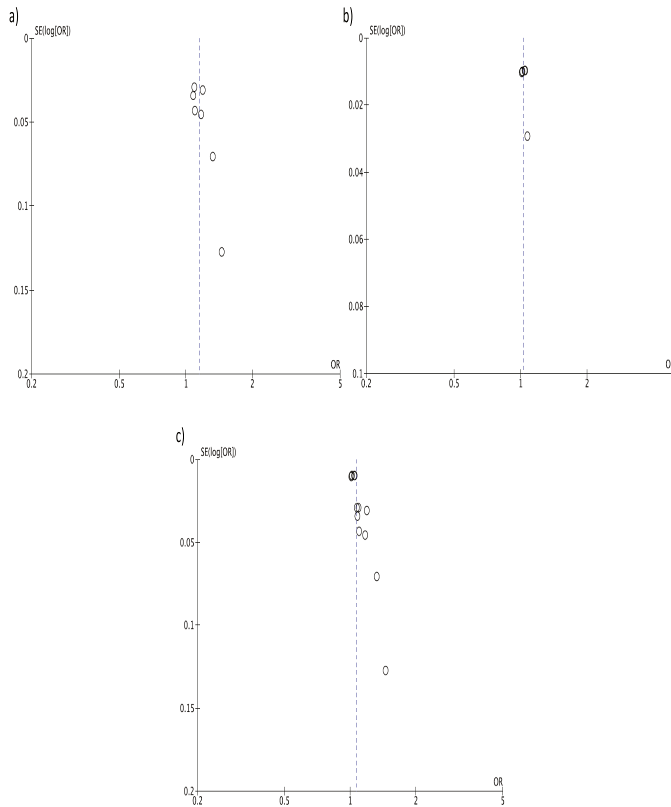


Figure 5. Funnel plots for colorectal cancer risk of a one-point increase of Dietary Inflammatory Index (DII): (**a**) case-control, (**b**) prospective, and (**c**) total studies.

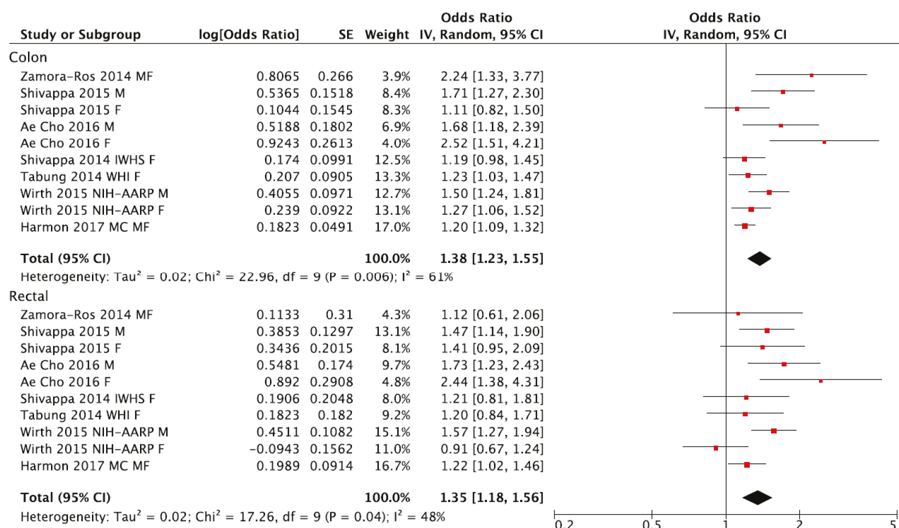


Figure 6. Forest plot of summary relative risks (RRs) of colon and rectal cancer for the highest *versus* lowest (reference) category of Dietary Inflammatory Index (DII).

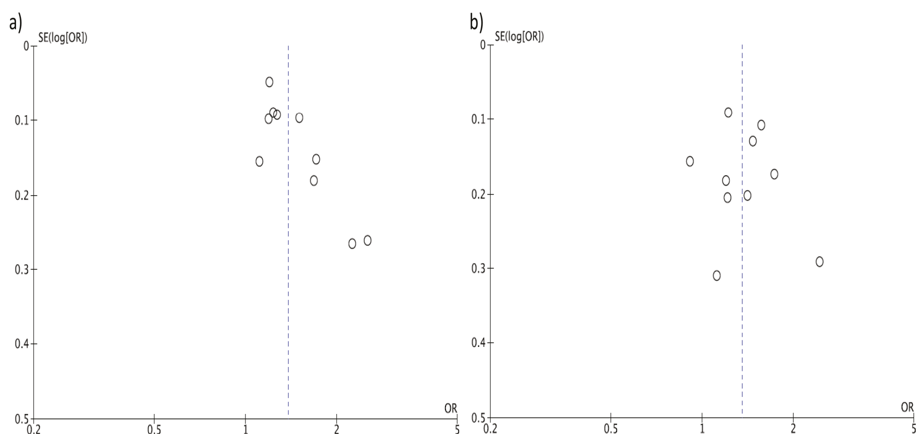


Figure 7. Funnel plots for colon and rectal cancer risk of the highest *versus* lowest (reference) category of Dietary Inflammatory Index (DII): (**a**) colon and (**b**) rectal.

4. Discussion

Results from this meta-analysis of nine studies that have examined the association between inflammatory potential of diet, as measured by the DII, and CRC, showed strong evidence of positive association between the DII and CRC. This persisted across tumor location. Therefore, limiting consumption of pro-inflammatory foods, such as red meat, and increasing consumption of anti-inflammatory components, like fruits and vegetables, may play an important role in reducing the risk of CRC. The DII score is calculated from several components and important among them are polyphenols such as flavonoids. Isoflavones, flavanol, flavan-3-ol, anthocyanidins, flavones and flavanones which form the six major groups of flavonoids are included in the DII calculation and all of these are anti-inflammatory and therefore have negative inflammatory effect scores [22].

The DII is a literature-derived population-based dietary index developed specifically to measure the inflammatory potential of individuals' overall diet across varying populations and dietary assessment methods [30]. There are other dietary indices that exist, such as The Healthy Eating Index (HEI) [51], Alternate Healthy Eating Index (AHEI) [52], Dietary Approaches to Stop Hypertension Score (DASH) [53] and Mediterranean Diet Score (MDS) [54]; and these indices have been examined with CRC as outcome in the past [55,56]. All of these indices represent a dietary scoring pattern that represents healthfulness of the diet. However, none was specifically developed to assess the diet's inflammatory potential. Another advantage of DII is that it is grounded in the peer-reviewed literature on diet and inflammation and is not dependent on a single study or a few studies within the same or similar populations. Rather, it is based on findings from nearly 2000 articles focusing on laboratory and human studies—from all over the world, employing different study designs and dietary assessment methods. Articles were scored based on the direction of association observed in the article, for example, if in an article garlic significantly reduced levels of CRP then the article would get a score of -1; these articles were then weighted based on the study design. Human studies were given more weight, and clinical trials were assigned the maximum weight of 10. The complete description on the design and development of DII is described in the DII development paper [22]. Findings from the Energy Balance Study indicated that the DII score was negatively correlated with the HEI-2010 ($r = -0.65$, $p < 0.01$), AHEI ($r = -0.55$, $p < 0.01$), and the DASH ($r = -0.52$, $p < 0.01$) [57] and in the Melbourne Collaborative Cohort Study, an inverse correlation was observed between the DII and MDS ($r = -0.45$, $p < 0.01$) [58]. Apart from showing a consistent association between DII and CRC, the DII also was successfully validated with inflammatory markers in several studies across different populations [24,28,29,59,60]. This suggests that the DII represents unique aspects of diet that go beyond what constitutes a generally healthy diet by capturing the specific effect of inflammation compared to other dietary indices. These results provide evidence that the DII is unique in its ability to relate specifically to the core issue of chronic inflammation.

There are several theories to explain the association between the DII and CRC risk; one of the most commonly considered is the effect of pro-inflammatory diet on insulin resistance through increasing systemic inflammation [61]. Another theory suggests the role of diet on local inflammation and oxidation in the colon, which results in focal proliferation and mutagenesis [62]. On the other hand, antioxidant compounds contained in key foods (i.e., fruits, vegetables, coffee, tea, etc.) may exert anti-inflammatory effects, especially locally through the action of local microbiota [63]. Although, we have observed strong evidence of association between DII scores and CRC, two studies examining the association between DII scores and the prevalence or recurrence of colorectal adenoma, which is a precursor of CRC, have produced equivocal results. One, conducted in the in the screening arm of the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, produced positive results, primarily in men [64]. The other, based on data from the Wheat Bran Fiber (WBF) and Ursodeoxycholic Acid (UDCA) Phase III clinical trials, produced null results [65]. Although colorectal adenoma is a risk factor for CRC, most adenomas will not undergo malignant transformation [66–68]. More studies are warranted to further understand this association.

Dietary factors can be related to CRC through mechanisms other than inflammation. For example, consumption of red and processed meat increases the risk of CRC through increased levels of the haem iron content [69], N-nitroso compounds formed during the processing of meat [70], of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines from cooking meat at high temperatures [71]. On the other hand, higher fibre intake is believed to be related to a lower risk of colorectal cancer via increase stool bulk, increase stool transit time, and dilute faecal carcinogens [72]. We have looked at the relative effects of the DII score versus other indices such as the healthy eating Index. Usually about 25 to 50% of the variability in one index is explained by the DII [57,73]. So, there clearly are other; that is, not inflammation-related, mechanisms that are operative. It is important to note that the DII would encompass the effect of haem iron, because iron is a pro-inflammatory component of the DII. Factors

related to the other effects of fiber are, indeed, related to inflammation. These other mechanisms, along with inflammation, may exacerbate the effect of diet on CRC.

This meta-analysis had some limitations. First, DII score for all the studies was based on self-reports collected from food frequency questionnaires, which are not error-free. We suspect that self-assessments using these instruments in case-control studies are encumbered by recall bias [74], which can lead to a potential misclassification of the exposure. Even in prospective studies such reports may be subject to response set biases [75,76]. Second, DII score was estimated at baseline and diets might change during study follow-up. However, adult dietary habits seem to be relatively stable over time [77]. It has also been shown in the Women's Health Initiative, where DII was measured at different time points, that changes in DII towards a pro-inflammatory diets are associated with an elevated risk of colon cancer [78]. Third, we observed substantial heterogeneity across studies pooling the CRC risk. The probable reasons for this could be the differences in the number of food parameters considered in the DII score in different studies, demographic characteristics, type of study, and follow-up duration (in the case of prospective studies).

5. Conclusions

In conclusion, this meta-analysis suggests that a more pro-inflammatory diet, as estimated by the higher DII score, was independently associated with an increased risk of CRC. Hence, promoting diets low in pro-inflammatory items and rich in anti-inflammatory food components should help in reducing the incidence of CRC. Future research should concentrate on how DII fares in a population with CRC and what effect it would have on CRC-specific mortality.

Acknowledgments: M.D.W., N.S. and J.R.H. were supported by grant number R44DK103377 from NIH's National Institute of Diabetes and Digestive and Kidney Diseases.

Author Contributions: N.S. and G.G. conceived and designed the experiments; J.G. performed the analyses; N.S., J.G. and G.G. wrote the first draft of the manuscript; J.R.H., M.W., G.P. and A.F.S. significantly contributed to the development of the final draft of the manuscript. All authors approve of the final version of the manuscript.

Conflicts of Interest: Disclosure: J.R.H. owns controlling interest in Connecting Health Innovations LLC (CHI), a company planning to license the right to his invention of the dietary inflammatory index (DII) from the University of South Carolina in order to develop computer and smart phone applications for patient counseling and dietary intervention in clinical settings. M.D.W. and N.S. are employees of CHI.

References

1. IARC Globocan 2012: Estimated Cancer Incidence, Mortality and Prevalance Worldwide 2012. Available online: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx (accessed on 18 September 2017).
2. Vogel, V.G.; McPherson, R.S. Dietary epidemiology of colon cancer. *Hematol. Oncol. Clin. N. Am.* **1989**, *3*, 35–63.
3. Keibel, A.; Singh, V.; Sharma, M.C. Inflammation, microenvironment, and the immune system in cancer progression. *Curr. Pharm. Des.* **2009**, *15*, 1949–1955. [CrossRef] [PubMed]
4. Pan, M.H.; Lai, C.S.; Dushenkov, S.; Ho, C.T. Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem.* **2009**, *57*, 4467–4477. [CrossRef] [PubMed]
5. Coussens, L.M.; Werb, Z. Inflammation and cancer. *Nature* **2002**, *420*, 860–867. [CrossRef] [PubMed]
6. Philip, M.; Rowley, D.A.; Schreiber, H. Inflammation as a tumor promoter in cancer induction. *Semin. Cancer Biol.* **2004**, *14*, 433–439. [CrossRef] [PubMed]
7. Chung, Y.-C.; Chang, Y.-F. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *J. Surg. Oncol.* **2003**, *83*, 222–226. [CrossRef] [PubMed]
8. Terzic, J.; Grivennikov, S.; Karin, E.; Karin, M. Inflammation and colon cancer. *Gastroenterology* **2010**, *138*, 2101–2114. [CrossRef] [PubMed]
9. Toriola, A.T.; Cheng, T.Y.; Neuhouser, M.L.; Wener, M.H.; Zheng, Y.; Brown, E.; Miller, J.W.; Song, X.; Beresford, S.A.A.; Gunter, M.J.; et al. Biomarkers of inflammation are associated with colorectal cancer risk in women but are not suitable as early detection markers. *Int. J. Cancer* **2013**, *132*, 2648–2658. [CrossRef] [PubMed]

10. Santos, S.; Oliveira, A.; Lopes, C. Systematic review of saturated fatty acids on inflammation and circulating levels of adipokines. *Nutr. Res.* **2013**, *33*, 687–695. [CrossRef] [PubMed]
11. Bordoni, A.; Danesi, F.; Dardevet, D.; Dupont, D.; Fernandez, A.S.; Gille, D.; dos Santos, C.N.; Pinto, P.; Re, R.; Rémond, D.; et al. Dairy products and inflammation: A review of the clinical evidence. *Crit. Rev. Food Sci. Nutr.* **2015**, *57*, 2497–2525. [CrossRef] [PubMed]
12. Barbaresko, J.; Koch, M.; Schulze, M.B.; Nothlings, U. Dietary pattern analysis and biomarkers of low-grade inflammation: A systematic literature review. *Nutr. Rev.* **2013**, *71*, 511–527. [CrossRef] [PubMed]
13. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **2002**, *56*, 365–379. [CrossRef]
14. Continuous Update Project Report. Food, Nutrition, Physical Activity, and the Prevention of Colorectal Cancer. Available online: http://www.aicr.org/assets/docs/pdf/reports/Second_Expert_Report.pdf (accessed on 18 September 2017).
15. Vuong, Q.V. Epidemiological evidence linking tea consumption to human health: a review. *Crit. Rev. food Sci. Nutr.* **2014**, *54*, 523–536. [CrossRef] [PubMed]
16. Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, Caffeine, and Health Outcomes: An Umbrella Review. *Annu. Rev. Nutr.* **2017**, in press. [CrossRef] [PubMed]
17. Zhang, C.; Qin, Y.Y.; Wei, X.; Yu, F.F.; Zhou, Y.H.; He, J. Tea consumption and risk of cardiovascular outcomes and total mortality: A systematic review and meta-analysis of prospective observational studies. *Eur. J. Epidemiol.* **2015**, *30*, 103–113. [CrossRef] [PubMed]
18. Grosso, G.; Micek, A.; Godos, J.; Sciacca, S.; Pajak, A.; Martinez-Gonzalez, M.A.; Giovannucci, E.L.; Galvano, F. Coffee consumption and risk of all-cause, cardiovascular, and cancer mortality in smokers and non-smokers: A dose-response meta-analysis. *Eur. J. Epidemiol.* **2016**, *31*, 1191–1205. [CrossRef] [PubMed]
19. Grosso, G.; Bella, F.; Godos, J.; Sciacca, S.; Del Rio, D.; Ray, S.; Galvano, F.; Giovannucci, E.L. Possible role of diet in cancer: Systematic review and multiple meta-analyses of dietary patterns, lifestyle factors, and cancer risk. *Nutr. Rev.* **2017**, in press. [CrossRef]
20. Godos, J.; Bella, F.; Torrisi, A.; Sciacca, S.; Galvano, F.; Grosso, G. Dietary patterns and risk of colorectal adenoma: A systematic review and meta-analysis of observational studies. *J. Hum. Nutr. Diet.* **2016**, *29*, 757–767. [CrossRef] [PubMed]
21. Cavicchia, P.P.; Steck, S.E.; Hurley, T.G.; Hussey, J.R.; Ma, Y.; Ockene, I.S.; Hébert, J.R. A new dietary inflammatory index predicts interval changes in high-sensitivity c-reactive protein. *J. Nutr.* **2009**, *139*, 2365–2372. [CrossRef] [PubMed]
22. Shivappa, N.; Steck, S.E.; Hurley, T.G.; Hussey, J.R.; Hebert, J.R. Designing and developing a literature-derived, population-based dietary inflammatory index. *Public Health Nutr.* **2014**, *17*, 1689–1696. [CrossRef] [PubMed]
23. Shivappa, N.; Steck, S.E.; Hurley, T.G.; Hussey, J.R.; Ma, Y.; Ockene, I.S.; Tabung, F.; Hebert, J.R. A population-based dietary inflammatory index predicts levels of C-reactive protein in the Seasonal Variation of Blood Cholesterol Study (SEASONS). *Public Health Nutr.* **2014**, *17*, 1825–1833. [CrossRef] [PubMed]
24. Tabung, F.K.; Steck, S.E.; Zhang, J.; Ma, Y.; Liese, A.D.; Agalliu, I.; Hingle, M.; Hou, L.; Hurley, T.G.; Jiao, L.; et al. Construct validation of the dietary inflammatory index among postmenopausal women. *Ann. Epidemiol.* **2015**, *25*, 398–405. [CrossRef] [PubMed]
25. Shivappa, N.; Hebert, J.R.; Marcos, A.; Diaz, L.E.; Gomez, S.; Nova, E.; Michels, N.; Arouca, A.; Gonzalez-Gross, M.; Castillo, M.J.; et al. Association between dietary inflammatory index and inflammatory markers in the HELENA study. *Mol. Nutr. Food Res.* **2017**. [CrossRef] [PubMed]
26. Shivappa, N.; Wirth, M.D.; Hurley, T.G.; Hebert, J.R. Association between the dietary inflammatory index (DII) and telomere length and C-reactive protein from the National Health and Nutrition Examination Survey-1999-2002. *Mol. Nutr. Food Res.* **2017**, *61*, 4. [CrossRef] [PubMed]
27. Vahid, F.; Shivappa, N.; Hekmatdoost, A.; Hebert, J.R.; Davoodi, S.H.; Sadeghi, M. Association between Maternal Dietary Inflammatory Index (DII) and abortion in Iranian women and validation of DII with serum concentration of inflammatory factors: Case-control study. *Appl. Physiol. Nutr. Metab.* **2017**, *42*, 511–516. [CrossRef] [PubMed]
28. Wood, L.G.; Shivappa, N.; Berthon, B.S.; Gibson, P.G.; Hebert, J.R. Dietary inflammatory index is related to asthma risk, lung function and systemic inflammation in asthma. *Clin. Exp. Allergy* **2015**, *45*, 177–183. [CrossRef] [PubMed]

29. Wirth, M.D.; Shivappa, N.; Davis, L.; Hurley, T.G.; Ortaglia, A.; Drayton, R.; Blair, S.N.; Hébert, J.R. Construct Validation of the Dietary Inflammatory Index among African Americans. *J. Nutr. Health Aging* **2017**, *21*, 487–491. [CrossRef] [PubMed]
30. Wirth, M.D.; Burch, J.; Shivappa, N.; Violanti, J.M.; Burchfiel, C.M.; Fekedulegn, D.; Andrew, M.E.; Hartley, T.A.; Miller, D.B.; Mnatsakanova, A.; et al. Association of a dietary inflammatory index with inflammatory indices and metabolic syndrome among police officers. *J. Occup. Environ. Med./Am. Coll. Occup. Environ. Med.* **2014**, *56*, 986–989. [CrossRef] [PubMed]
31. Shivappa, N.; Hebert, J.R.; Polesel, J.; Zucchetto, A.; Crispo, A.; Montella, M.; Franceschi, S.; Rossi, M.; La Vecchia, C.; Serraino, D. Inflammatory potential of diet and risk for hepatocellular cancer in a case-control study from Italy. *Br. J. Nutr.* **2016**, *115*, 324–331. [CrossRef] [PubMed]
32. Shivappa, N.; Hebert, J.R.; Rosato, V.; Rossi, M.; Montella, M.; Serraino, D.; La Vecchia, C. Dietary inflammatory index and ovarian cancer risk in a large Italian case-control study. *Cancer Causes Control* **2016**, *27*, 897–906. [CrossRef] [PubMed]
33. Shivappa, N.; Hebert, J.R.; Rosato, V.; Serraino, D.; La Vecchia, C. Inflammatory potential of diet and risk of laryngeal cancer in a case-control study from Italy. *Cancer Causes Control* **2016**, *27*, 1027–1034. [CrossRef] [PubMed]
34. Graffouillere, L.; Deschasaux, M.; Mariotti, F.; Neufcourt, L.; Shivappa, N.; Hebert, J.R.; Wirth, M.D.; Latino-Martel, P.; Hercberg, S.; Galan, P.; et al. Prospective association between the Dietary Inflammatory Index and mortality: Modulation by antioxidant supplementation in the SU.VI.MAX randomized controlled trial. *Am. J. Clin. Nutr.* **2016**, *103*, 878–885. [CrossRef] [PubMed]
35. Shivappa, N.; Blair, C.K.; Prizment, A.E.; Jacobs, D.R., Jr.; Steck, S.E.; Hebert, J.R. Association between inflammatory potential of diet and mortality in the Iowa Women's Health study. *Eur. J. Nutr.* **2016**, *55*, 1491–1502. [CrossRef] [PubMed]
36. Shivappa, N.; Steck, S.E.; Hussey, J.R.; Ma, Y.; Hebert, J.R. Inflammatory potential of diet and all-cause, cardiovascular, and cancer mortality in national health and nutrition examination survey iii study. *Eur. J. Nutr.* **2015**, *56*, 683–692. [CrossRef] [PubMed]
37. Maisonneuve, P.; Shivappa, N.; Hebert, J.R.; Bellomi, M.; Rampinelli, C.; Bertolotti, R.; Spaggiari, L.; Palli, D.; Veronesi, G.; Gnagnarella, P. Dietary inflammatory index and risk of lung cancer and other respiratory conditions among heavy smokers in the cosmos screening study. *Eur. J. Nutr.* **2016**, *55*, 1069–1079. [CrossRef] [PubMed]
38. Kesse-Guyot, E.; Assmann, K.E.; Andreeva, V.A.; Touvier, M.; Neufcourt, L.; Shivappa, N.; Hebert, J.R.; Wirth, M.D.; Hercberg, S.; Galan, P.; et al. Long-term association between the dietary inflammatory index and cognitive functioning: Findings from the su.Vi.Max study. *Eur. J. Nutr.* **2017**, *56*, 1647–1655. [CrossRef] [PubMed]
39. Sanchez-Villegas, A.; Ruiz-Canela, M.; de la Fuente-Arrillaga, C.; Gea, A.; Shivappa, N.; Hebert, J.R.; Martinez-Gonzalez, M.A. Dietary inflammatory index, cardiometabolic conditions and depression in the seguimiento universidad de navarra cohort study. *Br. J. Nutr.* **2015**, *114*, 1471–1479. [CrossRef] [PubMed]
40. Shivappa, N.; Hebert, J.R.; Steck, S.E.; Hofseth, L.J.; Shehadah, I.; Bani-Hani, K.E.; Al-Jaberi, T.; Al-Nusairr, M.; Heath, D.; Tayyem, R. Dietary inflammatory index and odds of colorectal cancer in a case-control study from Jordan. *Appl. Physiol. Nutr. Metab.* **2017**, *42*, 744–749. [CrossRef] [PubMed]
41. Harmon, B.E.; Wirth, M.D.; Boushey, C.J.; Wilkens, L.R.; Draluck, E.; Shivappa, N.; Steck, S.E.; Hofseth, L.; Haiman, C.A.; Le Marchand, L.; et al. The dietary inflammatory index is associated with colorectal cancer risk in the multiethnic cohort. *J. Nutr.* **2017**, *147*, 430–438. [CrossRef] [PubMed]
42. Wirth, M.D.; Shivappa, N.; Steck, S.E.; Hurley, T.G.; Hebert, J.R. The dietary inflammatory index is associated with colorectal cancer in the national institutes of health-american association of retired persons diet and health study. *Br. J. Nutr.* **2015**, *113*, 1819–1827. [CrossRef] [PubMed]
43. Tabung, F.K.; Steck, S.E.; Ma, Y.; Liese, A.D.; Zhang, J.; Caan, B.; Hou, L.; Johnson, K.C.; Mossavar-Rahmani, Y.; Shivappa, N.; et al. The association between dietary inflammatory index and risk of colorectal cancer among postmenopausal women: Results from the women's health initiative. *Cancer Causes Control* **2015**, *26*, 399–408. [CrossRef] [PubMed]
44. Zamora-Ros, R.; Shivappa, N.; Steck, S.E.; Canzian, F.; Landi, S.; Alonso, M.H.; Hebert, J.R.; Moreno, V. Dietary inflammatory index and inflammatory gene interactions in relation to colorectal cancer risk in the bellvitge colorectal cancer case-control study. *Genes Nutr.* **2015**, *10*, 447. [CrossRef] [PubMed]

45. Shivappa, N.; Prizment, A.E.; Blair, C.K.; Jacobs, D.R., Jr.; Steck, S.E.; Hebert, J.R. Dietary inflammatory index and risk of colorectal cancer in the iowa women's health study. *Cancer Epidemiol. Prev. Biomark.* **2014**, *23*, 2383–2392. [CrossRef] [PubMed]
46. Cho, Y.A.; Lee, J.; Oh, J.H.; Shin, A.; Kim, J. Dietary inflammatory index and risk of colorectal cancer: A case-control study in Korea. *Nutrients* **2016**, *8*, 469. [CrossRef] [PubMed]
47. Sharma, I.; Wang, P.P.; Zhu, Y.; Woodrow, J.R.; Mulay, S.; Parfrey, P.S.; McLaughlin, J.R.; Hebert, J.R.; Shivappa, N.; Li, Y.; et al. Inflammatory diet and risk of colorectal cancer: A population based case-control study in newfoundland, Canada. *Nutrition* **2017**. [CrossRef] [PubMed]
48. Shivappa, N.; Zucchetto, A.; Montella, M.; Serraino, D.; Steck, S.E.; La Vecchia, C.; Hebert, J.R. Inflammatory potential of diet and risk of colorectal cancer: A case-control study from italy. *Br. J. Nutr.* **2015**, *114*, 152–158. [CrossRef] [PubMed]
49. Wells GA, S.B.; O'Connell, D.; Peterson, J.; Welch, V.; Losos, M.; Tugwell, P. *The Newcastle-Ottawa Scale (nos) for Assessing the Quality of Nonrandomised Studies in Meta-Analyses*; Ottawa Health Research Institute: Ottawa, ON, Canada, 1999.
50. Greenland, S. Quantitative methods in the review of epidemiologic literature. *Epidemiol. Rev.* **1987**, *9*, 1–30. [CrossRef] [PubMed]
51. Kennedy, E.T.; Ohls, J.; Carlson, S.; Fleming, K. The healthy eating index: Design and applications. *J. Am. Diet. Assoc.* **1995**, *95*, 1103–1108. [CrossRef]
52. McCullough, M.L.; Feskanich, D.; Stampfer, M.J.; Giovannucci, E.L.; Rimm, E.B.; Hu, F.B.; Spiegelman, D.; Hunter, D.J.; Colditz, G.A.; Willett, W.C. Diet quality and major chronic disease risk in men and women: Moving toward improved dietary guidance. *Am. J. Clin. Nutr.* **2002**, *76*, 1261–1271. [PubMed]
53. Fung, T.T.; Chiuve, S.E.; McCullough, M.L.; Rexrode, K.M.; Logroscino, G.; Hu, F.B. Adherence to a dash-style diet and risk of coronary heart disease and stroke in women. *Arch. Intern. Med.* **2008**, *168*, 713–720. [CrossRef] [PubMed]
54. Panagiotakos, D.B.; Pitsavos, C.; Stefanadis, C. Dietary patterns: A mediterranean diet score and its relation to clinical and biological markers of cardiovascular disease risk. *Nutr. Metab. Cardiovasc. Dis.* **2006**, *16*, 559–568. [CrossRef] [PubMed]
55. Park, S.Y.; Boushey, C.J.; Wilkens, L.R.; Haiman, C.A.; Le Marchand, L. High-quality diets associate with reduced risk of colorectal cancer: Analyses of diet quality indexes in the multiethnic cohort. *Gastroenterology* **2017**. [CrossRef] [PubMed]
56. Vargas, A.J.; Neuhouser, M.L.; George, S.M.; Thomson, C.A.; Ho, G.Y.; Rohan, T.E.; Kato, I.; Nassir, R.; Hou, L.; Manson, J.E. Diet quality and colorectal cancer risk in the women's health initiative observational study. *Am. J. Epidemiol.* **2016**, *184*, 23–32. [CrossRef] [PubMed]
57. Wirth, M.D.; Hebert, J.R.; Shivappa, N.; Hand, G.A.; Hurley, T.G.; Drenowatz, C.; McMahon, D.; Shook, R.P.; Blair, S.N. Anti-inflammatory dietary inflammatory index scores are associated with healthier scores on other dietary indices. *Nutr. Res.* **2016**, *36*, 214–219. [CrossRef] [PubMed]
58. Hodge, A.M.; Bassett, J.K.; Shivappa, N.; Hebert, J.R.; English, D.R.; Giles, G.G.; Severi, G. Dietary inflammatory index, mediterranean diet score, and lung cancer: A prospective study. *Cancer Causes Control* **2016**, *27*, 907–917. [CrossRef] [PubMed]
59. Ruiz-Canela, M.; Zazpe, I.; Shivappa, N.; Hebert, J.R.; Sanchez-Tainta, A.; Corella, D.; Salas-Salvado, J.; Fito, M.; Lamuela-Raventos, R.M.; Rekondo, J.; et al. Dietary inflammatory index and anthropometric measures of obesity in a population sample at high cardiovascular risk from the predimed (prevencion con dieta mediterranea) trial. *Br. J. Nutr.* **2015**, *113*, 984–995. [CrossRef] [PubMed]
60. Julia, C.; Assmann, K.E.; Shivappa, N.; Hebert, J.R.; Wirth, M.D.; Hercberg, S.; Touvier, M.; Kesse-Guyot, E. Long-term associations between inflammatory dietary scores in relation to long-term c-reactive protein status measured 12 years later: Findings from the supplementation en vitamines et mineraux antioxydants (su.Vi.Max) cohort. *Br. J. Nutr.* **2017**, *117*, 306–314. [CrossRef] [PubMed]
61. Festa, A.; D'Agostino, R.; Howard, G.; Mykkanen, L.; Tracy, R.P.; Haffner, S.M. Chronic subclinical inflammation as part of the insulin resistance syndrome—The insulin resistance atherosclerosis study (IRAS). *Circulation* **2000**, *102*, 42–47. [CrossRef] [PubMed]
62. Bruce, W.R.; Giacca, A.; Medline, A. Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidem. Biomar.* **2000**, *9*, 1271–1279.

63. Grosso, G.; Godos, J.; Lamuela-Raventos, R.; Ray, S.; Micek, A.; Pajak, A.; Sciacca, S.; D'Orazio, N.; Rio, D.D.; Galvano, F. A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. *Mol. Nutr. Food Res.* **2017**. [CrossRef] [PubMed]
64. Haslam, A.; Wagner Robb, S.; Hebert, J.R.; Huang, H.; Wirth, M.D.; Shivappa, N.; Ebell, M.H. The association between dietary inflammatory index scores and the prevalence of colorectal adenoma. *Public Health Nutr.* **2017**, *20*, 1609–1616. [CrossRef] [PubMed]
65. Sardo Molmenti, C.L.; Steck, S.E.; Thomson, C.A.; Hibler, E.A.; Yang, J.; Shivappa, N.; Greenlee, H.; Wirth, M.D.; Neugut, A.I.; Jacobs, E.T.; et al. Dietary inflammatory index and risk of colorectal adenoma recurrence: A pooled analysis. *Nutr. Cancer* **2017**, *69*, 238–247. [CrossRef] [PubMed]
66. Chen, C.D.; Yen, M.F.; Wang, W.M.; Wong, J.M.; Chen, T.H. A case-cohort study for the disease natural history of adenoma-carcinoma and de novo carcinoma and surveillance of colon and rectum after polypectomy: Implication for efficacy of colonoscopy. *Br. J. Cancer* **2003**, *88*, 1866–1873. [CrossRef] [PubMed]
67. Xirasagar, S.; Li, Y.-T.; Burch, J.B.; Daguise, V.; Hurley, T.G.; Hebert, J.R. Reducing colorectal cancer incidence and disparities: Performance and outcomes of a screening colonoscopy program in south carolina. *Adv. Public Health* **2014**. [CrossRef] [PubMed]
68. Xirasagar, S.; Hurley, T.G.; Sros, L.; Hebert, J.R. Quality and safety of screening colonoscopies performed by primary care physicians. *Med. Care* **2010**, *48*, 703–709. [CrossRef] [PubMed]
69. Gilsing, A.M.; Fransen, F.; de Kok, T.M.; Goldbohm, A.R.; Schouten, L.J.; de Bruine, A.P.; van Engeland, M.; van den Brandt, P.A.; de Goeij, A.F.; Weijenberg, M.P. Dietary heme iron and the risk of colorectal cancer with specific mutations in KRAS and APC. *Carcinogenesis* **2013**, *34*, 2757–2766. [CrossRef] [PubMed]
70. Zhu, Y.; Wang, P.P.; Zhao, J.; Green, R.; Sun, Z.; Roebothan, B.; Squires, J.; Buehler, S.; Dicks, E.; Zhao, J.; et al. Dietary n-nitroso compounds and risk of colorectal cancer: A case-control study in newfoundland and labrador and ontario, canada. *Br. J. Nutr.* **2014**, *111*, 1109–1117. [CrossRef] [PubMed]
71. Diggs, D.L.; Huderson, A.C.; Harris, K.L.; Myers, J.N.; Banks, L.D.; Rekhadevi, P.V.; Niaz, M.S.; Ramesh, A. Polycyclic aromatic hydrocarbons and digestive tract cancers: A perspective. *J. Environ. Sci. Health Part C* **2011**, *29*, 324–357. [CrossRef] [PubMed]
72. Young, G.P.; Hu, Y.; Le Leu, R.K.; Nyskohus, L. Dietary fibre and colorectal cancer: A model for environment—Gene interactions. *Mol. Nutr. Food Res.* **2005**, *49*, 571–584. [CrossRef] [PubMed]
73. Navarro, S.L.; Neuhouser, M.L.; Cheng, T.D.; Tinker, L.F.; Shikany, J.M.; Snetselaar, L.; Martinez, J.A.; Kato, I.; Beresford, S.A.; Chapkin, R.S.; et al. The interaction between dietary fiber and fat and risk of colorectal cancer in the women's health initiative. *Nutrients* **2016**, *8*, 779. [CrossRef] [PubMed]
74. Gittelsohn, J.; Shankar, A.V.; Pokhrel, R.P.; West, K.P., Jr. Accuracy of estimating food intake by observation. *J. Am. Diet. Assoc.* **1994**, *94*, 1273–1277. [CrossRef]
75. Hebert, J.R.; Ebbeling, C.B.; Matthews, C.E.; Hurley, T.G.; Ma, Y.; Druker, S.; Clemow, L. Systematic errors in middle-aged women's estimates of energy intake: Comparing three self-report measures to total energy expenditure from doubly labeled water. *Anna. Epidemiol.* **2002**, *12*, 577–586. [CrossRef]
76. Hebert, J.R.; Ma, Y.; Clemow, L.; Ockene, I.S.; Saperia, G.; Stanek, E.J., III; Merriam, P.A.; Ockene, J.K. Gender differences in social desirability and social approval bias in dietary self-report. *Am. J. Epidemiol.* **1997**, *146*, 1046–1055. [CrossRef] [PubMed]
77. Thompson, F.E.; Metzner, H.L.; Lamphiear, D.E.; Hawthorne, V.M. Characteristics of individuals and long term reproducibility of dietary reports: The tecumseh diet methodology study. *J. Clin. Epidemiol.* **1990**, *43*, 1169–1178. [CrossRef]
78. Tabung, F.K.; Steck, S.E.; Ma, Y.; Liese, A.D.; Zhang, J.; Lane, D.S.; Ho, G.Y.F.; Hou, L.; Snetselaar, L.; Ockene, J.K.; et al. Changes in the inflammatory potential of diet over time and risk of colorectal cancer in postmenopausal women. *Am. J. Epidemiol.* **2017**. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Biological Mechanisms by Which Antiproliferative Actions of Resveratrol Are Minimized

Yih Ho [1], Yu-Syuan Lin [1,2], Hsuan-Liang Liu [3], Ya-Jung Shih [2,4], Shin-Ying Lin [2,4], Ai Shih [5], Yu-Tang Chin [2], Yi-Ru Chen [6,†], Hung-Yun Lin [2,4,7,*] and Paul J. Davis [7,8]

1 School of Pharmacy, College of Pharmacy, Pharmacy School, Taipei Medical University, Taipei 11031, Taiwan; yiho@tmu.edu.tw (Y.H.); oaykee@gmail.com (Y.-S.L.)
2 Taipei Cancer Center, Taipei Medical University, Taipei 11031, Taiwan; yajungshih@yahoo.com.tw (Y.-J.S.); cindylin812@yahoo.com.tw (S.-Y.L.); yutangchin@gmail.com (Y.-T.C.)
3 Department of Chemical Engineering and Biotechnology, Institute of Biochemical and Biomedical Engineering, National Taipei University of Technology, Taipei 10608, Taiwan; f10894@ntut.edu.tw
4 Ph.D. Program for Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei 11031, Taiwan
5 National Laboratory Animal Center, Taipei 11599, Taiwan; ashih@narlabs.org.tw
6 Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 11529, Taiwan; aquarlus9132@gmail.com
7 Pharmaceutical Research Institute, Albany College of Pharmacy and Health Sciences, Rensselaer, NY 12208, USA; pdavis.ordwayst@gmail.com
8 Department of Medicine, Albany Medical College, Albany, NY 12208, USA
* Correspondence: linhy@tmu.edu.tw; Tel.: +886-2-2736-1661
† Current address: Taipei Cancer Center, Ph.D. Program for Cancer Biology and Drug Discovery, Taipei Medical University, Taipei 11031, Taiwan.

Received: 10 August 2017; Accepted: 17 September 2017; Published: 21 September 2017

Abstract: Preclinical and clinical studies have offered evidence for protective effects of various polyphenol-rich foods against cardiovascular diseases, neurodegenerative diseases, and cancers. Resveratrol is among the most widely studied polyphenols. However, the preventive and treatment effectiveness of resveratrol in cancer remain controversial because of certain limitations in existing studies. For example, studies of the activity of resveratrol against cancer cell lines in vitro have often been conducted at concentrations in the low μM to mM range, whereas dietary resveratrol or resveratrol-containing wine rarely achieve nM concentrations in the clinic. While the mechanisms underlying the failure of resveratrol to inhibit cancer growth in the intact organism are not fully understood, the interference by thyroid hormones with the anticancer activity of resveratrol have been well documented in both in vitro and xenograft studies. Thus, endogenous thyroid hormones may explain the failure of anticancer actions of resveratrol in intact animals, or in the clinic. In this review, mechanisms involved in resveratrol-induced antiproliferation and effects of thyroid hormones on these mechanisms are discussed.

Keywords: resveratrol; antiproliferation; cancer; tetrac; Nano-diamino-tetrac

1. Introduction

Clinical evaluation of resveratrol and other well-studied polyphenols as anticancer agents have at best yielded variable results [1–10]. It is also important to point out that meta-analyses of clinical studies that have described the anticancer activity of polyphenols have not been based on prospective studies, but on a case–control study design, as noted by Grosso and co-workers [5].

The lack of congruence in preclinical and clinical studies of resveratrol and other polyphenols may reflect a low bioavailability for the administered compounds, a limited effectiveness of the metabolites, and the systemic toxicity that has served to limit dosing [2,3,11]. In addition, concentrations of the

unconjugated form of resveratrol usually employed in in vitro studies are not obtained in vivo in tumor tissue [12] because of dose ceilings that are in place. Thus, much of the in vitro experience with resveratrol and other polyphenols is not relevant to clinical practice. Incomplete understanding of the mechanisms of the actions of resveratrol in cancer settings, in the intact animal or patient, may also be limiting our ability to translate preclinical results into clinical cancer management [2]. Bridging the translational gap between preclinical and clinical applications of resveratrol may be facilitated by monitoring circulating biomarkers of tissue action of resveratrol at various doses [13]. Such markers are insulin-like growth factor-1 (IGF-1) or IGF binding proteins.

In the current review, we describe certain anticancer functions of resveratrol, observed in vitro or in other preclinical studies, as they are reduced or blocked entirely by factors that come into play in the intact animal. We will point out that some of these anti-resveratrol actions are subject to modulation, with the result of restoring anticancer resveratrol effects.

2. Resveratrol Has Been Shown to Have Potential in Cancer Prevention and as an Antiproliferative Agent in Cancer

Resveratrol (3,4′,5-trihydroxystilbene), one of the best-studied stilbenes, exists principally in grapes, blueberries, peanuts, pistachios, and hops, and red wine also contains substantial amounts of resveratrol [14,15]. Resveratrol exists in both *cis*- and *trans*-stereoisomeric forms and it is the *trans*-isomer that is biologically active [15,16]. Preclinical studies have shown that resveratrol has cancer-preventive activity against various stages of development, for most cancers, including prostate, breast, stomach, colon, lung, thyroid, and pancreas [17]. Evidence also indicates that resveratrol is able to inhibit cancer cell proliferation and to induce cell cycle arrest and apoptosis in various types of cancer cells [18–20]. In addition, resveratrol induces differentiation in certain cell types [21–23].

The loss of mitochondrial membrane potential has been described as an early response to resveratrol that is relevant to apoptosis, and resveratrol treatment in isolated mitochondria has led to depolarization, suggesting that the drug may target mitochondria directly [24]. Resveratrol suppresses the activation of nuclear factor κB (NFκB) by upregulating mitogen-activated protein kinase (MAPK)-phosphatase-5 [25,26]; this serves to reduce the induction by NFκB of the expression of various anti-apoptosis genes [27]. The resveratrol-containing crude extract of seeds from Melinjo fruit (*Gnetum gnemon* L.) has been reported to induce apoptosis in cancer cells via caspase-3/7-dependent and caspase-independent mechanisms [28]. On the other hand, gnetin C, a resveratrol dimer, and active ingredient of seeds from Melinjo, can trigger both early- and late-stage apoptosis in cancer cells, at least in part by activating caspase 3/7-dependent mechanisms [28]. Resveratrol also induces the release of cytochrome c and Smac/Diablo from mitochondria, and subsequently, the activation of caspase-9 (4–8-fold) and caspase-3 (4–6-fold) after depolarization of the mitochondria [24].

The expression of matrix metalloproteinase (MMP) genes as a part of tumor invasion and metastasis is inhibited by resveratrol. The latter also decreases vascular endothelial growth factor (VEGF) levels in cancer cells, thus supporting anti-angiogenesis. Resveratrol has also been reported to sensitize cancer cells to ionizing radiation [29–31].

Prostaglandins are a product of cyclooxygenase (COX-2) action on arachidonic acid and have been shown to stimulate cell proliferation, promote angiogenesis, and suppress apoptosis—all of which support malignancy [32–34]. Resveratrol inhibits inflammation by directly blocking COX-2 activity. However, it was recently shown that resveratrol has another critical effect on COX-2, namely the induction of tumor cell antiproliferation via activated ERK1/2-dependent nuclear accumulation of COX-2 and activation/phosphorylation of p53-dependent apoptosis, in breast cancer, glioma, head and neck squamous cell cancer, and ovarian cancer cells [35–38].

The proposed mechanism for the antiproliferation induced by resveratrol is depicted in Figure 1. Via specific binding to the cancer cell surface integrin, αvβ3, resveratrol sequentially induces the activation of ERK1/2, nuclear localization of sumoylated COX-2, phosphorylation of p53, and antiproliferation. A specific COX-2 inhibitor, NS-398, is able to inhibit the complexing of nuclear COX-2 and activated

ERK1/2, but does not affect resveratrol-induced ERK1/2 activation. Both ERK1/2 activation and nuclear COX-2 accumulation are required to complex with activated p53, to form a co-activator complex for p53-responsive genes, and for p53-dependent apoptosis in resveratrol-treated cells [39].

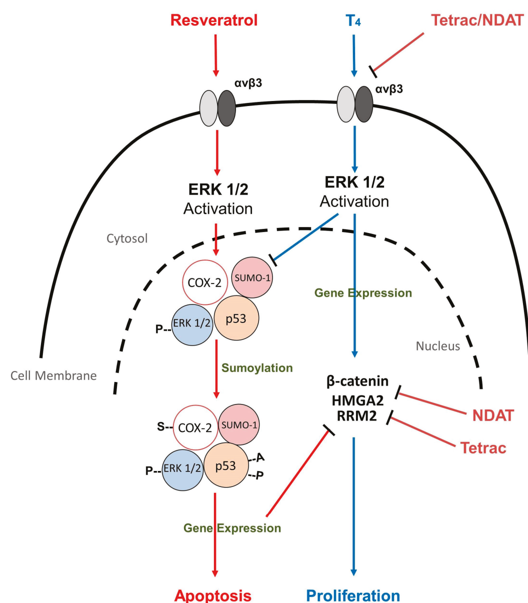


Figure 1. Thyroid hormone-induced interference with resveratrol-induced antiproliferation is blocked by tetrac and its nanoparticulate analog. Signal transduction pathways are involved in resveratrol-induced antiproliferation and thyroid hormone-mediated proliferation in cancer cells. Thyroid hormones, as T_4, in physiological concentrations, stimulate cancer cell proliferation via the hormone receptor site on integrin αvβ3, on the cell surface. Activated ERK1/2 (pERK1/2) is required for hormone-dependent cell proliferation, as shown by the ERK1/2 cascade inhibition by the selective inhibitor, PD98059. A distinct receptor for the stilbene is also present on integrin αvβ3, through which resveratrol activates ERK1/2 and induces nuclear cyclooxygenase (COX-2) accumulation. Phosphorylated ERK1/2, induced by resveratrol, also translocates into the cell nucleus and complexes with inducible COX-2 in resveratrol-treated cancer cells. This is an essential upstream step in the induction by resveratrol of p53 phosphorylation at Ser-15, and consequent p53-dependent antiproliferation. Blocking resveratrol-induced nuclear accumulation of COX-2 inhibits p53 phosphorylation and antiproliferation. T_4 inhibits the formation of the intranuclear pERK1/2-COX-2–p53 complex and consequent p53-dependent antiproliferation. The mechanism by which T_4 inhibits the generation of pERK1/2–COX-2–p53 nuclear complexes in resveratrol-exposed cells is not yet known. Although the activation of ERK1/2 induced by hormones and resveratrol is additive, the competition for pools of ERK1/2 between thyroid hormones and resveratrol appears to divert kinases to the cell proliferation pathway, and may play an important role in the inhibition by T_4 of resveratrol's pro-apoptotic action. Tetrac inhibits the expression of β-catenin and HMGA2, and NDAT inhibits the expression of RRM2, which is caused by resveratrol. In addition, both tetrac and NDAT activate the expression of Chibby, which binds to β-catenin and blocks its transcriptional activities. Thus, tetrac or NDAT may restore pro-apoptotic activity of resveratrol that has been lost due to the actions of endogenous thyroid hormones. Abbreviations: COX-2, cyclooxygenase-2; HMGA2, high mobility group AT-hook 2; tetrac/NDAT, tetraiodothyroacetic acid/nano–diamino–tetrac; RRM2, ribonucleoside diphosphate reductase subunit M2; Chibby, nuclear protein that directly binds to β-catenin and antagonizes its transcriptional activity; SUMO-2, small ubiquitin-related modifier-2.

3. Inhibition by Resveratrol of Carcinogenesis in Animal Models

Resveratrol has limited bioavailability, but has been shown convincingly to prevent, and efficiently treat, tumors of the skin, esophagus, and gastrointestinal tract [40]. Different anticancer cellular mechanisms for resveratrol have been proposed, as noted above, and these include the induction of apoptosis and the inhibition of tumor-related angiogenesis [41,42]. Outcomes of such studies routinely reveal that the xenograft volumes and weights of resveratrol groups are less than those of control groups, with little or no systemic toxicity, (e.g., the net body mass of resveratrol groups in xenografted mice is not significantly different from that of control groups ($p > 0.05$) [43]).

At clinically achievable concentrations, the Melinjo (*Gnetum gnemon* L.) seed extract (MSE) and gnetin C mentioned above, significantly inhibit the proliferation of various cancer cells via apoptosis, without affecting normal cells. Gnetin C also has significantly greater antitumor activity than resveratrol [28]. Oral administration of MSE at 50 and 100 mg/kg per day significantly blocks tumor growth, induces intra-tumoral angiogenesis, and significantly reduces liver metastases in BALB/c mice bearing colon-26 tumors [28].

Resveratrol decreases tumor cell viability in vitro by 75% to 90%, primarily by the induction of apoptosis [24], and can inhibit tumor growth by more than 80% in vivo [24]. The apoptotic index of resveratrol-treated groups in animals is significantly higher than control groups. The expression of p53 and ERK activation are simultaneously and significantly increased in resveratrol-treated groups [43]. In NOD/SCID (non-obese diabetic/severe combined immune-deficient) mice xenografted with breast cancer stem-like cells (BCSC), resveratrol treatment (100 mg/kg/d) inhibited tumor growth. Analyses of the expression of autophagy-relevant LC3-II, Beclin1 and *Atg 7* genes in BCSCs indicated that resveratrol induced autophagy [44]. Resveratrol also suppressed the activity of the Wnt/β-catenin signaling pathway in BCSCs. Induction of an overexpression of β-catenin in BCSCs markedly decreased the cytotoxicity and autophagy caused by resveratrol in BCSCs [44].

Disadvantages of Resveratrol

The pharmacokinetic and metabolite profiles of resveratrol have been defined in patients, e.g., those with colon cancer [12]. Resveratrol administered to healthy volunteers confirmed that it is well-tolerated and modulates enzyme systems involved in carcinogen activation and detoxification [45]. While it has promising anticancer potential, resveratrol has limited bioavailability, and thus efficacy is seen to be limited to tumors to which the agent can be directly applied, e.g., skin cancer and gastrointestinal tract tumors. It inhibits tumor growth effectively when it is administered intratumorally. The serum concentrations of resveratrol achieved in human subjects are in the low μM range (2–10 μM/L) [24]. Interestingly, there is no accumulation of resveratrol itself observed in tumor tissue [24], but metabolites, such as resveratrol glucuronide and piceatannol, are found in serum, liver, skin, and xenografted tumor tissue [46].

An optimal dose or dosing schedule for resveratrol has not been defined clinically. There is insufficient clinical evidence to validate a recommendation for the prophylactic administration of resveratrol [47]. For example, athymic mice received control diets or diets containing 110 μM or 263 μM of resveratrol, 2 weeks prior to a subcutaneous injection of melanoma cells. Resveratrol was rapidly metabolized, and at any concentration tested, resveratrol failed to inhibit human melanoma xenograft growth [46]. The higher levels of resveratrol tested (0.006% in food or 100 mg in slow-release pellets) tended, paradoxically, to stimulate tumor growth ($p = 0.08–0.09$) [44]. Piceatannol is a stilbenoid—a resveratrol-like compound—that does not affect the in vitro growth of a murine melanoma cell line, but stimulates the number of lung metastases significantly when these melanoma cells are directly injected into the tail veins of mice [46]. Thus, the effects of phytochemicals on cultured cells, and in the intact animal, may be inconsistent [46]. The foregoing information that is derived from a variety of tumor cells makes it difficult to devise and recommend additional clinical trials for resveratrol in solid tumors of any type [48].

Acute lymphoblastic leukemia (ALL) with translocation t(4;11) is a high-risk leukemia that is found in 60–85% of infants with ALL. It is often refractory to conventional chemotherapeutics after relapse. The efficacy of dietary resveratrol was examined in vivo in a model of the human t(4;11) ALL cell line SEM engrafted by tail vein injection into 5-week-old NOD.CB17-Prkdcscid/J mice, fed for 3 weeks with a regular diet or a diet containing 0.2% w/w resveratrol. Compared with the regular diet, dietary resveratrol did not postpone engraftment of the leukemia cells. Dietary resveratrol did not sensitize leukemic cells to vincristine, an antileukemic agent active in metaphase and an inducer of apoptosis [49].

4. Resveratrol-Induced Antiproliferation Is Opposed by Circulating Thyroid Hormone

Thyroid hormones play vital roles in normal physiological activities. However, studies have shown that thyroid hormones are able to interact with resveratrol when the latter is added in vitro or in vivo. Several interactions are listed in Table 1. The inhibition of cancer cells by thyroid hormones, through resveratrol-induced antiproliferation, has been studied in detail by our group. This action on cancer cells by thyroid hormones is mediated by a cell surface receptor for the hormone on integrin αvβ3 [50]. T_3 (3,3′,5-Triiodo-L-thyronine) is responsible for the intracellular effects of thyroid hormones in normal cells, whereas L-thyroxine (T_4)—usually viewed exclusively as a prohormone for T_3—is the principal thyroid hormone ligand of the receptor on the plasma membrane of cancer cells. The physiological circulating concentration of free T_3 is about 10^{-11} M and we have found this level of T_3 to have no effect on resveratrol-induced antiproliferation. In contrast, circulating levels of free T_4 do oppose the anticancer effects of resveratrol. The same cell surface protein (integrin αvβ3) has receptors for both thyroid hormones, and for resveratrol and other small molecules, but the binding sites are distinct from one another and do not interact on the integrin [51]. Agonist ligands of αvβ3 activate intracellular pools of MAPK (ERK1/2) and such activation has downstream (intracellular) consequences for thyroid hormones and resveratrol.

Thyroid hormones, particularly T_4, play a key role in cancer progression [52–57]. In addition, thyroid hormones can overlap with the cellular functions of estrogen, enhancing cancer cell proliferation via nuclear estrogen receptor-α (ERα) Ser-118 phosphorylation in human ERα-positive cancer cells [56,58]. Thus, the signal transduction pathways of estrogen-stimulated ERα-positive cancer cell proliferation and that of thyroid hormones, are identical [58].

T_4 inhibits the expression of antiproliferative BAD (Bcl-2-associated death promoter) and induces the expression of proliferative *BCL2* in malignant cells [59]. Thyroid hormones also control the expression of other *BCL2* gene family members, which have anti-apoptotic consequences. In addition, thyroid hormones decrease cellular abundances of caspases—such as caspase-3 and BAX, the gene product of which is pro-apoptotic in mitochondria [59]. Thus, the hormone is anti-apoptotic. Thyroid hormones can also prevent apoptosis damage in hypothyroid rat liver cells, induced by oxidative stress at the inner mitochondrial membrane [60]. The hormones induce the expression of myeloid cell leukemia 1 (MCL1), a principal Bcl-2-related protein that resides in the outer mitochondrial membrane, thus preventing mitochondrial membrane destabilization and the formation of channels by which the release of mitochondrial cytochrome c occurs, also leading to apoptosis [61]. T_4 also increases the expression of *XIAP* (X-linked inhibitor of apoptosis) [59] and of HIF-1α [53,62]. Both have been shown to play an important role in anti-apoptotic functions. Chemotherapeutic agents with pro-apoptotic properties, such as paclitaxel, etoposide, and doxorubicin are subject to exportation from cells by the P-glycoprotein (P-gp, MDR1 or ABCB1) membrane *pump* [63]. The expression of the *MDR1* gene and the function of the *pump* gene product have been shown to be induced by thyroid hormones. Thyroid hormone-induced intracellular alkalization and Na^+/H^+ exchanger (NHE1) may also be related to the function of MDR1 [60,63]. Thus, thyroid hormones activate a panel of anti-apoptotic mechanisms.

Table 1. Studies that document interactions of thyroid hormones with resveratrol.

Study Design	Exposure/Result	Reference
Evaluation of resveratrol for its protective effects against fluoride-induced metabolic dysfunctions in the rat thyroid gland	Subacute exposure to sodium fluoride (dose of 20 mg/kg bw/day orally for 30 days) induced thyroidal dysfunction	[64]
Assessment of the effects of subcutaneous (s.c.) and oral administration of 17β-estradiol (E_2) and the actions of resveratrol on the pituitary–thyroid axis in ovariectomized (OVX) female rats for 3 months	In vitro and in vivo studies demonstrated that serum resveratrol levels of 1.0 and 8.1 μM led to significant increases in total serum triiodthyronine (T_3) levels. Ovariectomy induced thyroid stimuating hormone-β (TSHβ) mRNA expression in the adenohypophysis and E_2 administration attenuated this effect. Treatment of OVX rats with s.c. E_2 implants did not affect the pituitary–thyroid axis, whereas oral E_2 benzoate (E_2B) increased plasma TSH and total thyroxine (T_4)	[65]
Assessment of the possibility that thyroid hormones are anti-apoptotic	In vitro, T_4 induced ERK1/2 activation and caused minimal Ser-15 phosphorylation of p53. However, T_4 did not affect the c-fos, c-jun and p21 mRNA abundances in proliferating human papillary and follicular thyroid cancer cells; cell proliferation was reduced by resveratrol co-incubation.	[55]
Examined the mechanism whereby T_4 inhibits resveratrol-induced apoptosis in glioma cells	In vitro, T_4 inhibited resveratrol-induced nuclear COX-2 and cytosolic pro-apoptotic protein (BcLx-s) accumulation. T_4 inhibited resveratrol-induced apoptosis by interfering with the interaction of nuclear COX-2 and ERK1/2. T_4 and resveratrol bind to discrete sites on integrin αvβ3.	[54]
Effects of treatment, with varying doses of resveratrol, on medullary thyroid cancer	In vitro, resveratrol treatment resulted in suppression of cell proliferation and increased cleavage of caspase-3 and poly(ADP-ribose)polymerase (PARP). A dose-dependent decrease in the abundance of ASCL1, a neuroedocrine transcription factor, was observed at protein and mRNA levels. CgA, a protein marker of hormone secretion, was also reduced. A dose-dependent induction of Notch2 mRNA was observed (qPCR).	[66]
Examination of the ability of polyphenol phytochemicals (including resveratrol) to induce redifferentiation in thyroid cancer cell lines.	The cell lines—TPC-1, FTC-133, NPA, FRO, and ARO—exhibited growth inhibition in response to resveratrol. Resveratrol decreased CD97 expression in FTC-133, NPA, and FRO thyroid cancer cell lines; there was increased expression of the differentiation marker, NIS, in FTC-133 cells, but no change in NPA, FRO, and ARO cells. Findings suggested that resveratrol may provide a useful therapeutic intervention in thyroid cancer redifferentiation therapy	[67]

These actions of thyroid hormones are rapidly initiated at the binding site for thyroid hormones on αvβ3. Thyroxine, at a physiological concentration, is able to interfere with resveratrol-induced antiproliferation by inhibiting serine phosphorylation of p53 and blocking resveratrol-induced cancer cell apoptosis [35]. The intracellular consequences downstream of the integrin-activated ERK1/2, for resveratrol at its receptor, and for thyroid hormones at their separate binding sites, are functionally opposed. Resveratrol induces nuclear COX-2 accumulation by an undefined mechanism, although resveratrol-induced sumoylation of COX-2 may be involved. In contrast, thyroxine prevents the accumulation of nuclear COX-2 in cancer cells and T_4, in addition, blocks the formation of the nuclear complex of pERK1/2 and COX-2, which is induced by resveratrol in cancer cells [54].

RGD (Arg–Gly–Asp) peptide, an integrin αvβ3 antagonist, with its own recognition site, has been shown to inhibit certain functions of the integrin binding site for thyroid hormones and of the receptor for resveratrol [53].

Tetraiodothyroacetic acid (tetrac), the deaminated analog of T_4, can inhibit thyroid hormone-induced activities by blocking the interaction of thyroid hormones with the cell surface integrin αvβ3 receptor. Tetrac does not affect resveratrol-induced biological activity [50,68], but thyroxine-induced inhibitory effects on the resveratrol-induced nuclear COX-2 accumulation, and later p53 phosphorylation, are prevented by tetrac [54]. How these signal transduction kinase steps are differentially regulated by T_4 and resveratrol, is not yet clear. In addition to blocking the binding of thyroid hormones to integrin αvβ3, tetrac and a nanoparticulate derivative, nano–diamino–tetrac (NDAT), inhibit cancer cell proliferation [69] and block angiogenesis by disrupting the expression of a number of genes relevant to apoptosis and blood vessel formation. Studies of xenografts conducted in our lab have shown that the anticancer effect induced by low concentrations of resveratrol can be potentiated by both tetrac and NDAT. Tetrac inhibits the expression of the genes for *β-catenin* and *high mobility group AT-hook 2* (*HMGA2*), and thus potentiates resveratrol-induced antiproliferation in colorectal cancer cells. In addition, NDAT inhibits the expression of the ribonucleoside diphosphate reductase subunit *M2* (*RRM2*) gene, which is induced by resveratrol, but interferes with resveratrol-induced antiproliferation. The NDAT effect on *RRM2* thus facilitates resveratrol-induced antiproliferation. Both of these potentiating effects by tetrac and NDAT on resveratrol-induced anticancer growth are observed in xenografts of colorectal cancer HCT116 cells.

5. Conclusions

Resveratrol, via binding to a specific receptor on plasma membrane integrin αvβ3, induces ERK1/2- and p53-dependent antiproliferation in cancer cells. The induction of antiproliferation by resveratrol also depends upon the accumulation of resveratrol-induced nuclear COX-2, complexed with ERK1/2, and consequently with p53, to form a complex with transcriptional activity. Various endogenous circulating hormones affect cancer cell proliferation, metastasis, and invasion. The interactions between hormones and anticancer drugs have been intensively studied. We emphasize here that physiological concentrations of thyroid hormones, particularly T_4, interfere with the well-described antiproliferative activity of resveratrol. Thyroid hormones and resveratrol bind to discrete receptors on plasma membrane integrin (αvβ3), to induce the activation of discrete intracellular pools of ERK1/2 in cancer cells, by a yet undefined mechanism. The ERK1/2 activated by thyroid hormones interestingly blocks ERK1/2-dependent formation of resveratrol-induced, p53-requiring nucleoprotein complexes; this action inhibits the antiproliferative effect of the polyphenol. In the xenografted animal model, and in the clinic, circulating physiological levels of thyroid hormones may be responsible for the suppression of resveratrol-induced anticancer activities in clinical studies. We suggest that the clinical efficacy of resveratrol in the treatment of cancer may be restored or potentiated by blocking the cancer cell surface receptor for thyroid hormone in vivo, or by reducing circulating levels of T_4 and substituting T_3 to maintain euthyroidism (euthyroid hypothyroxinemia) [70].

Acknowledgments: This study is dedicated to the memory of Faith B. Davis for her endless support and extraordinary editorial skill to our research and papers. Appreciation is sent to Ya-Jung Shih, Yi-Ru Chen, and Shin-Ying Lin for their endless contribution of studies described in this review article, in addition to artistic work on the schema by Ya-Jung and ABu. Animals used in our studies described here were supplied by National Laboratory Animal Center, Taiwan. Works from our laboratory described in this review article were supported in part by a collaborating grant of TMU and National Taipei University of Technology (USTP-NTUT-TMU-104-09), a grant from Ministry of Education (TMU Top University Fund), and grants from Ministry of Science and Technology, Taiwan (MOST 104-2314-B-038 -046 -MY3; MOST105-2320-B-038-006).

Author Contributions: Y.H., Y.-S.L., H.-L.L., H.-Y.L. and P.J.D. conceived and designed the structure of the manuscript; Y.-J.S., A.S., Y.-T.C. and Y.-R.C. searched and reorganized references; Y.H., Y.-S.L. and Y.-T.C. analyzed the data from the references; Y.H., Y.-J.S. and Y.-R.C. drew the Figure, and H.-Y.L. wrote the manuscript. All authors reviewed the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. McCubrey, J.A.; Lertpiriyapong, K.; Steelman, L.S.; Abrams, S.L.; Yang, L.V.; Murata, R.M.; Rosalen, P.L.; Scalisi, A.; Neri, L.M.; Cocco, L.; et al. Effects of resveratrol, curcumin, berberine and other nutraceuticals on aging, cancer development, cancer stem cells and micrornas. *Aging* **2017**, *9*, 1477–1536. [CrossRef] [PubMed]
2. Maru, G.B.; Hudlikar, R.R.; Kumar, G.; Gandhi, K.; Mahimkar, M.B. Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. *World J. Biol. Chem.* **2016**, *7*, 88–99. [CrossRef] [PubMed]
3. Davatgaran-Taghipour, Y.; Masoomzadeh, S.; Farzaei, M.H.; Bahramsoltani, R.; Karimi-Soureh, Z.; Rahimi, R.; Abdollahi, M. Polyphenol nanoformulations for cancer therapy: Experimental evidence and clinical perspective. *Int. J. Nanomed.* **2017**, *12*, 2689–2702. [CrossRef] [PubMed]
4. Kotecha, R.; Takami, A.; Espinoza, J.L. Dietary phytochemicals and cancer chemoprevention: A review of the clinical evidence. *Oncotarget* **2016**, *7*, 52517–52529. [CrossRef] [PubMed]
5. Grosso, G.; Godos, J.; Lamuela-Raventos, R.; Ray, S.; Micek, A.; Pajak, A.; Sciacca, S.; D'Orazio, N.; Del Rio, D.; Galvano, F. A comprehensive meta-analysis on dietary flavonoid and lignan intake and cancer risk: Level of evidence and limitations. *Mol. Nutr. Food Res.* **2017**, *61*, 1600930. [CrossRef] [PubMed]
6. Chang, B.; Sang, L.; Wang, Y.; Tong, J.; Wang, B.Y. Consumption of tea and risk for pancreatic cancer: A meta-analysis of published epidemiological studies. *Nutr. Cancer* **2014**, *66*, 1109–1123. [CrossRef] [PubMed]
7. Yuan, J.M. Cancer prevention by green tea: Evidence from epidemiologic studies. *Am. J. Clin. Nutr.* **2013**, *98*, 1676S–1681S. [CrossRef] [PubMed]
8. Boehm, K.; Borrelli, F.; Ernst, E.; Habacher, G.; Hung, S.K.; Milazzo, S.; Horneber, M. Green tea (*Camellia sinensis*) for the prevention of cancer. *Cochrane Database Syst. Rev.* **2009**, CD005004. [CrossRef]
9. Vang, O.; Ahmad, N.; Baile, C.A.; Baur, J.A.; Brown, K.; Csiszar, A.; Das, D.K.; Delmas, D.; Gottfried, C.; Lin, H.Y.; et al. What is new for an old molecule? Systematic review and recommendations on the use of resveratrol. *PLoS ONE* **2011**, *6*, e19881. [CrossRef] [PubMed]
10. Shrubsole, M.J.; Lu, W.; Chen, Z.; Shu, X.O.; Zheng, Y.; Dai, Q.; Cai, Q.; Gu, K.; Ruan, Z.X.; Gao, Y.T.; et al. Drinking green tea modestly reduces breast cancer risk. *J. Nutr.* **2009**, *139*, 310–316. [CrossRef] [PubMed]
11. Guthrie Ariane, R. Effects of resveratrol on drug and carcinogenmetabolizing enzymes, implications for cancer prevention. *Pharmacol. Res. Perspect.* **2017**, *5*, e00294. [CrossRef] [PubMed]
12. Patel, K.R.; Brown, V.A.; Jones, D.J.; Britton, R.G.; Hemingway, D.; Miller, A.S.; West, K.P.; Booth, T.D.; Perloff, M.; Crowell, J.A.; et al. Clinical pharmacology of resveratrol and its metabolites in colorectal cancer patients. *Cancer Res.* **2010**, *70*, 7392–7399. [CrossRef] [PubMed]
13. Brown, V.A.; Patel, K.R.; Viskaduraki, M.; Crowell, J.A.; Perloff, M.; Booth, T.D.; Vasilinin, G.; Sen, A.; Schinas, A.M.; Piccirilli, G.; et al. Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: Safety, pharmacokinetics, and effect on the insulin-like growth factor axis. *Cancer Res.* **2010**, *70*, 9003–9011. [CrossRef] [PubMed]
14. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278. [CrossRef] [PubMed]
15. Catalgol, B.; Batirel, S.; Taga, Y.; Ozer, N.K. Resveratrol: French paradox revisited. *Front. Pharmacol.* **2012**, *17*, 141. [CrossRef] [PubMed]
16. Resveratrol. Monograph. *Altern. Med. Rev.* **2010**, *15*, 152–158.
17. Udenigwe, C.C.; Ramprasath, V.R.; Aluko, R.E.; Jones, P.J. Potential of resveratrol in anticancer and anti-inflammatory therapy. *Nutr. Rev.* **2008**, *66*, 445–454. [CrossRef] [PubMed]
18. Sheth, S.; Jajoo, S.; Kaur, T.; Mukherjea, D.; Sheehan, K.; Rybak, L.P.; Ramkumar, V. Resveratrol reduces prostate cancer growth and metastasis by inhibiting the Akt/MicroRNA-21 pathway. *PLoS ONE* **2012**, *7*, e51655. [CrossRef] [PubMed]
19. Slusarz, A.; Shenouda, N.S.; Sakla, M.S.; Drenkhahn, S.K.; Narula, A.S.; MacDonald, R.S.; Besch-Williford, C.L.; Lubahn, D.B. Common botanical compounds inhibit the hedgehog signaling pathway in prostate cancer. *Cancer Res.* **2010**, *70*, 3382–3390. [CrossRef] [PubMed]

20. Wang, T.T.; Hudson, T.S.; Wang, T.C.; Remsberg, C.M.; Davies, N.M.; Takahashi, Y.; Kim, Y.S.; Seifried, H.; Vinyard, B.T.; Perkins, S.N.; et al. Differential effects of resveratrol on androgen-responsive lncap human prostate cancer cells in vitro and in vivo. *Carcinogenesis* **2008**, *29*, 2001–2010. [CrossRef] [PubMed]
21. Wang, Y.; Romigh, T.; He, X.; Orloff, M.S.; Silverman, R.H.; Heston, W.D.; Eng, C. Resveratrol regulates the PTEN/AKT pathway through androgen receptor-dependent and -independent mechanisms in prostate cancer cell lines. *Hum. Mol. Genet.* **2010**, *19*, 4319–4329. [CrossRef] [PubMed]
22. Wang, Q.; Li, H.; Wang, X.W.; Wu, D.C.; Chen, X.Y.; Liu, J. Resveratrol promotes differentiation and induces Fas-independent apoptosis of human medulloblastoma cells. *Neurosci. Lett.* **2003**, *351*, 83–86. [CrossRef] [PubMed]
23. Gehm, B.D.; McAndrews, J.M.; Chien, P.Y.; Jameson, J.L. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 14138–14143. [CrossRef] [PubMed]
24. Van Ginkel, P.R.; Sareen, D.; Subramanian, L.; Walker, Q.; Darjatmoko, S.R.; Lindstrom, M.J.; Kulkarni, A.; Albert, D.M.; Polans, A.S. Resveratrol inhibits tumor growth of human neuroblastoma and mediates apoptosis by directly targeting mitochondria. *Clin. Cancer Res.* **2007**, *13*, 5162–5169. [CrossRef] [PubMed]
25. Bishayee, A.; Dhir, N. Resveratrol-mediated chemoprevention of diethylnitrosamine-initiated hepatocarcinogenesis: Inhibition of cell proliferation and induction of apoptosis. *Chem. Biol. Interact.* **2009**, *15*, 131–144. [CrossRef] [PubMed]
26. Hudson, T.S.; Hartle, D.K.; Hursting, S.D.; Nunez, N.P.; Wang, T.T.Y.; Young, H.A.; Arany, P.; Green, J.E. Inhibition of prostate cancer growth by muscadine grape skin extract and resveratrol through distinct mechanisms. *Cancer Res.* **2007**, *67*, 8396–8405. [CrossRef] [PubMed]
27. Xia, Y.; Shen, S.; Verma, I.M. NF-κB, an active player in human cancers. *Cancer Immunol. Res.* **2014**, *2*, 823–830. [CrossRef] [PubMed]
28. Narayanan, N.K.; Kunimasa, K.; Yamori, Y.; Mori, M.; Mori, H.; Nakamura, K.; Miller, G.; Manne, U.; Tiwari, A.K.; Narayanan, B. Antitumor activity of melinjo (*Gnetum gnemon* L.) seed extract in human and murine tumor models in vitro and in a colon-26 tumor-bearing mouse model in vivo. *Cancer Med.* **2015**, *4*, 1767–1780. [CrossRef] [PubMed]
29. Hsieh, T.C. Antiproliferative effects of resveratrol and the mediating role of resveratrol targeting protein NQO2 in androgen receptor-positive, hormone-non-responsive CWR22Rv1 cells. *Anticancer Res.* **2009**, *29*, 3011–3017. [PubMed]
30. Rashid, A.; Liu, C.; Sanli, T.; Tsiani, E.; Singh, G.; Bristow, R.G.; Dayes, I.; Lukka, H.; Wright, J.; Tsakiridis, T. Resveratrol enhances prostate cancer cell response to ionizing radiation. Modulation of the AMPK, Akt and mTOR pathways. *Radiat. Oncol.* **2011**, *6*, 144. [CrossRef] [PubMed]
31. Nelson, W.G.; De Marzo, A.M.; DeWeese, T.L.; Isaacs, W.B. The role of inflammation in the pathogenesis of prostate cancer. *J. Urol.* **2004**, *172*, S6–S11. [CrossRef] [PubMed]
32. De la Lastra, C.A.; Villegas, I. Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. *Mol. Nutr. Food Res.* **2005**, *49*, 405–430. [CrossRef] [PubMed]
33. Tsujii, M.; DuBois, R.N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* **1995**, *83*, 493–501. [CrossRef]
34. Tsujii, M.; Kawano, S.; Tsuji, S.; Sawaoka, H.; Hori, M.; DuBois, R.N. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **1998**, *93*, 705–716. [CrossRef]
35. Lin, H.Y.; Sun, M.; Tang, H.Y.; Simone, T.M.; Wu, Y.H.; Grandis, J.R.; Cao, H.J.; Davis, P.J.; Davis, F.B. Resveratrol causes COX-2- and p53-dependent apoptosis in head and neck squamous cell cancer cells. *J. Cell Biochem.* **2008**, *104*, 2131–2142. [CrossRef] [PubMed]
36. Yang, S.H.; Lin, H.Y.; Changou, C.A.; Chen, C.H.; Liu, Y.R.; Wang, J.; Jiang, X.; Luh, F.; Yen, Y. Integrin β3 and LKB1 are independently involved in the inhibition of proliferation by lovastatin in human intrahepatic cholangiocarcinoma. *Oncotarget* **2016**, *7*, 362–373. [CrossRef] [PubMed]
37. Hercbergs, A.H.; Lin, H.Y.; Davis, F.B.; Davis, P.J.; Leith, J.T. Radiosensitization and production of DNA double-strand breaks in U87MG brain tumor cells induced by tetraiodothyroacetic acid (tetrac). *Cell Cycle* **2011**, *10*, 352–357. [CrossRef] [PubMed]
38. Lin, H.Y.; Tang, H.Y.; Davis, F.B.; Davis, P.J. Resveratrol and apoptosis. *Ann. N. Y. Acad. Sci.* **2011**, *1215*, 79–88. [CrossRef] [PubMed]

39. Lin, H.Y.; Hsieh, M.T.; Cheng, G.Y.; Lai, H.Y.; Chin, Y.T.; Shih, Y.J.; Nana, A.W.; Lin, C.Y.; Yang, Y.C.; Tang, H.Y.; et al. Mechanisms of action of non-peptide hormones on resveratrol-induced anti-proliferation in cancer cells. *Ann. N. Y. Acad. Sci.* **2017**. [CrossRef] [PubMed]
40. Varoni, E.M.; Lo Faro, A.F.; Sharifi-Rad, J.; Iriti, M. Anticancer molecular mechanisms of resveratrol. *Front. Nutr.* **2016**, *3*, 8. [CrossRef] [PubMed]
41. Devipriya, S.; Ganapathy, V.; Shyamaladevi, C.S. Suppression of tumor growth and invasion in 9,10 dimethyl benz(*a*) anthracene induced mammary carcinoma by the plant bioflavonoid quercetin. *Chem.-Biol. Interact.* **2006**, *162*, 106–113. [CrossRef] [PubMed]
42. Bishayee, A. Cancer prevention and treatment with resveratrol: From rodent studies to clinical trials. *Cancer Prev. Res.* **2009**, *2*, 409–418. [CrossRef] [PubMed]
43. Hao, Y.; Huang, W.; Liao, M.; Zhu, Y.; Liu, H.; Hao, C.; Liu, G.; Zhang, G.; Feng, H.; Ning, X.; et al. The inhibition of resveratrol to human skin squamous cell carcinoma A431 xenografts in nude mice. *Fitoterapia* **2013**, *86*, 84–91. [CrossRef] [PubMed]
44. Fu, Y.; Chang, H.; Peng, X.; Bai, Q.; Yi, L.; Zhou, Y.; Zhu, J.; Mi, M. Resveratrol inhibits breast cancer stem-like cells and induces autophagy via suppressing Wnt/beta-catenin signaling pathway. *PLoS ONE* **2014**, *9*, e102535.
45. Chow, H.H.; Garland, L.L.; Hsu, C.H.; Vining, D.R.; Chew, W.M.; Miller, J.A.; Perloff, M.; Crowell, J.A.; Alberts, D.S. Resveratrol modulates drug- and carcinogen-metabolizing enzymes in a healthy volunteer study. *Cancer Prev. Res.* **2010**, *3*, 1168–1175. [CrossRef] [PubMed]
46. Niles, R.M.; Cook, C.P.; Meadows, G.G.; Fu, Y.M.; McLaughlin, J.L.; Rankin, G.O. Resveratrol is rapidly metabolized in athymic (nu/nu) mice and does not inhibit human melanoma xenograft tumor growth. *J. Nutr.* **2006**, *136*, 2542–2546. [PubMed]
47. Agrawal, D.K.; Mishra, P.K. Curcumin and its analogues: Potential anticancer agents. *Med. Res. Rev.* **2010**, *30*, 818–860. [CrossRef] [PubMed]
48. Klink, J.C.; Tewari, A.K.; Masko, E.M.; Antonelli, J.; Febbo, P.G.; Cohen, P.; Dewhirst, M.W.; Pizzo, S.V.; Freedland, S.J. Resveratrol worsens survival in SCID mice with prostate cancer xenografts in a cell-line specific manner, through paradoxical effects on oncogenic pathways. *Prostate* **2013**, *73*, 754–762. [CrossRef] [PubMed]
49. Zunino, S.J.; Storms, D.H.; Newman, J.W.; Pedersen, T.L.; Keen, C.L.; Ducore, J.M. Dietary resveratrol does not delay engraftment, sensitize to vincristine or inhibit growth of high-risk acute lymphoblastic leukemia cells in NOD/SCID mice. *Int. J. Oncol.* **2012**, *41*, 2207–2212. [CrossRef] [PubMed]
50. Lin, H.Y.; Chin, Y.T.; Yang, Y.C.; Lai, H.Y.; Wang-Peng, J.; Liu, L.F.; Tang, H.Y.; Davis, P.J. Thyroid hormone, cancer, and apoptosis. *Compr. Physiol.* **2016**, *6*, 1221–1237. [PubMed]
51. Freindorf, M.; Furlani, T.R.; Kong, J.; Cody, V.; Davis, F.B.; Davis, P.J. Combined QM/MM study of thyroid and steroid hormone analogue interactions with αvβ3 integrin. *J. Biomed. Biotechnol.* **2012**, *2012*, 959057. [CrossRef] [PubMed]
52. Davis, F.B.; Tang, H.Y.; Shih, A.; Keating, T.; Lansing, L.; Hercbergs, A.; Fenstermaker, R.A.; Mousa, A.; Mousa, S.A.; Davis, P.J.; et al. Acting via a cell surface receptor, thyroid hormone is a growth factor for glioma cells. *Cancer Res.* **2006**, *66*, 7270–7275. [CrossRef] [PubMed]
53. Lin, H.Y.; Sun, M.; Tang, H.Y.; Lin, C.; Luidens, M.K.; Mousa, S.A.; Incerpi, S.; Drusano, G.L.; Davis, F.B.; Davis, P.J. L-thyroxine vs. 3,5,3′-triiodo-L-thyronine and cell proliferation: Activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Am. J. Physiol. Cell Physiol.* **2009**, *296*, C980–C991. [CrossRef] [PubMed]
54. Lin, H.Y.; Tang, H.Y.; Keating, T.; Wu, Y.H.; Shih, A.; Hammond, D.; Sun, M.; Hercbergs, A.; Davis, F.B.; Davis, P.J. Resveratrol is pro-apoptotic and thyroid hormone is anti-apoptotic in glioma cells: Both actions are integrin and ERK mediated. *Carcinogenesis* **2008**, *29*, 62–69. [CrossRef] [PubMed]
55. Lin, H.Y.; Tang, H.Y.; Shih, A.; Keating, T.; Cao, G.; Davis, P.J.; Davis, F.B. Thyroid hormone is a MAPK-dependent growth factor for thyroid cancer cells and is anti-apoptotic. *Steroids* **2007**, *72*, 180–187. [CrossRef] [PubMed]
56. Meng, R.; Tang, H.Y.; Westfall, J.; London, D.; Cao, J.H.; Mousa, S.A.; Luidens, M.; Hercbergs, A.; Davis, F.B.; Davis, P.J.; et al. Crosstalk between integrin αvβ3 and estrogen receptor-α is involved in thyroid hormone-induced proliferation in human lung carcinoma cells. *PLoS ONE* **2011**, *6*, e27547. [CrossRef] [PubMed]

57. Yalcin, M.; Lin, H.Y.; Sudha, T.; Bharali, D.J.; Meng, R.; Tang, H.Y.; Davis, F.B.; Stain, S.C.; Davis, P.J.; Mousa, S.A. Response of human pancreatic cancer cell xenografts to tetraiodothyroacetic acid nanoparticles. *Horm. Cancer* **2013**, *4*, 176–185. [CrossRef] [PubMed]
58. Tang, H.Y.; Lin, H.Y.; Zhang, S.; Davis, F.B.; Davis, P.J. Thyroid hormone causes mitogen-activated protein kinase-dependent phosphorylation of the nuclear estrogen receptor. *Endocrinology* **2004**, *145*, 3265–3272. [CrossRef] [PubMed]
59. Davis, P.J.; Glinsky, G.V.; Lin, H.Y.; Leith, J.T.; Hercbergs, A.; Tang, H.Y.; Ashur-Fabian, O.; Incerpi, S.; Mousa, S.A. Cancer cell gene expression modulated from plasma membrane integrin αvβ3 by thyroid hormone and nanoparticulate tetrac. *Front. Endocrinol.* **2014**, *5*, 240.
60. Mukherjee, S.; Samanta, L.; Roy, A.; Bhanja, S.; Chainy, G.B. Supplementation of T_3 recovers hypothyroid rat liver cells from oxidatively damaged inner mitochondrial membrane leading to apoptosis. *Biomed. Res. Int.* **2014**, *2014*, 590897. [CrossRef] [PubMed]
61. Pietrzak, M.; Puzianowska-Kuznicka, M. Triiodothyronine utilizes phosphatidylinositol 3-kinase pathway to activate anti-apoptotic myeloid cell leukemia-1. *J. Mol. Endocrinol.* **2008**, *41*, 177–186. [CrossRef] [PubMed]
62. Lin, H.Y.; Su, Y.F.; Hsieh, M.T.; Lin, S.R.; Meng, R.; London, D.; Lin, C.; Tang, H.Y.; Hwang, J.; Davis, F.B.; et al. Nuclear monomeric integrin αv in cancer cells is a coactivator regulated by thyroid hormone. *FASEB J.* **2013**, *27*, 3209–3216. [CrossRef] [PubMed]
63. Davis, P.J.; Incerpi, S.; Lin, H.Y.; Tang, H.Y.; Sudha, T.; Mousa, S.A. Thyroid hormone and P-glycoprotein in tumor cells. *Biomed. Res. Int.* **2015**, *2015*, 168427. [CrossRef] [PubMed]
64. Sarkar, C.; Pal, S. Ameliorative effect of resveratrol against fluoride-induced alteration of thyroid function in male Wistar rats. *Biol. Trace Elem. Res.* **2014**, *162*, 278–287. [CrossRef] [PubMed]
65. Böttner, M.; Christoffel, J.; Rimoldi, G.; Wuttke, W. Effects of long-term treatment with resveratrol and subcutaneous and oral estradiol administration on the pituitary-thyroid-axis. *Exp. Clin. Endocrinol. Diabetes* **2006**, *114*, 82–90. [CrossRef] [PubMed]
66. Truong, M.; Cook, M.R.; Pinchot, S.N.; Kunnimalaiyaan, M.; Chen, H. Resveratrol induces Notch2-mediated apoptosis and suppression of neuroendocrine markers in medullary thyroid cancer. *Ann. Surg. Oncol.* **2011**, *18*, 1506–1511. [CrossRef] [PubMed]
67. Kang, H.J.; Youn, Y.K.; Hong, M.K.; Kim, L.S. Antiproliferation and redifferentiation in thyroid cancer cell lines by polyphenol phytochemicals. *J. Korean Med. Sci.* **2011**, *26*, 893–899. [CrossRef] [PubMed]
68. Davis, P.J.; Davis, F.B.; Mousa, S.A.; Luidens, M.K.; Lin, H.Y. Membrane receptor for thyroid hormone: Physiologic and pharmacologic implications. *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 99–115. [CrossRef] [PubMed]
69. Lin, H.Y.; Chin, Y.T.; Nana, A.W.; Shih, Y.J.; Lai, H.Y.; Tang, H.Y.; Leinung, M.; Mousa, S.A.; Davis, P.J. Actions of L-thyroxine and Nano-diamino-tetrac (Nanotetrac) on PD-L1 in cancer cells. *Steroids* **2016**, *114*, 59–67. [CrossRef] [PubMed]
70. Hercbergs, A.; Johnson, R.E.; Ashur-Fabian, O.; Garfield, D.H.; Davis, P.J. Medically induced euthyroid hypothyroxinemia may extend survival in compassionate need cancer patients: An observational study. *Oncologist* **2015**, *20*, 72–76. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Review

Therapeutic Effects of Olive and Its Derivatives on Osteoarthritis: From Bench to Bedside

Kok-Yong Chin [1,*] and Kok-Lun Pang [2]

1 Department of Pharmacology, Universiti Kebangsaan Malaysia Medical Centre, Cheras 56000, Malaysia
2 Biomedical Science Programme, School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur 50300, Malaysia; pangkoklun@gmail.com
* Correspondence: chinkokyong@ppukm.ukm.edu.my; Tel.: +60-16-708-2900

Received: 15 August 2017; Accepted: 20 September 2017; Published: 26 September 2017

Abstract: Osteoarthritis is a major cause of morbidity among the elderly worldwide. It is a disease characterized by localized inflammation of the joint and destruction of cartilage, leading to loss of function. Impaired chondrocyte repair mechanisms, due to inflammation, oxidative stress and autophagy, play important roles in the pathogenesis of osteoarthritis. Olive and its derivatives, which possess anti-inflammatory, antioxidant and autophagy-enhancing activities, are suitable candidates for therapeutic interventions for osteoarthritis. This review aimed to summarize the current evidence on the effects of olive and its derivatives, on osteoarthritis and chondrocytes. The literature on animal and human studies has demonstrated a beneficial effect of olive and its derivatives on the progression of osteoarthritis. In vitro studies have suggested that the augmentation of autophagy (though sirtuin-1) and suppression of inflammation by olive polyphenols could contribute to the chondroprotective effects of olive polyphenols. More research and well-planned clinical trials are required to justify the use of olive-based treatment in osteoarthritis.

Keywords: autophagy; cartilage; chondrocyte; hydroxytyrosol; inflammation; joint; oleocanthal; oleuropein; sirtuin-1; tyrosol

1. Introduction

Osteoarthritis (OA) is a common degenerative disease of the joints among the elderly. It is induced by accumulated micro- and macro-injuries that lead to a maladaptive repair response of the joints [1]. Current perspective holds that molecular derangements, characterised by abnormal joint metabolism, precede anatomical/physiological derangements of the joint, which are marked by cartilage degradation, increased subchondral bone remodelling, osteophyte formation and joint inflammation [1,2]. This ultimately translates to clinical manifestations of OA, which are marked by joint pain, tenderness, stiffness, impaired movement, crepitus and effusion [1,2]. The worldwide prevalence of knee OA is 3.8% (95% uncertainty interval (UI) 3.6% to 4.1%) and the prevalence of hip OA is 0.85% (95% UI 0.74% to 1.02%). Despite not being a fatal disease, it constituted the 11th largest cause of global disability (the 15th in 1990) and the 38th largest contributor of disability-adjusted life years (the 49th in 1990), in 2010 [3]. The annual mean direct and indirect medical costs of OA per patient were estimated to be €2013 (range €0.7–12), but this estimate was skewed towards Western countries [4].

Aging is the major risk factor for OA [5,6]. Under normal conditions, chondrocytes are responsible for the synthesis, regeneration and maintenance of the cartilage matrix. However, senescence of chondrocytes (chondrosenescence) occurs as a consequence of aging. This process compromises the ability of the chondrocytes to maintain and repair the articular cartilage tissue [5,6]. Chondrosenescence, with the prevailing harmful biomechanical stress, leads to irreversible chondrocyte cell death, which ultimately leads to cartilage damage, matrix depletion and loss of cartilage cellularity [7–10].

Several pathways are involved in the pathogenesis of OA. Numerous studies have reported the involvement of inflammation in the progression of OA. Mechanical damage can cause a localized inflammatory response of the joint, marked by increased pro-inflammatory mediators, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), nitrite oxide (NO) and prostaglandin E_2 (PGE_2) in the joint space [11–15]. This inflammatory response further exaggerates cartilage tissue damage via oxidative stress and damage, thus forming a vicious self-destructive cycle. Oxidative stress is also related to OA, as evidenced by an upregulation of inducible NO synthase (iNOS) and nicotinamide adenine dinucleotide phosphate oxidase in chondrocytes [6]. These enzymes produce high levels of reactive oxygen and nitrogen species (ROS and RNS), including NO, superoxide anion, peroxynitrite and hydrogen peroxide (H_2O_2) [16–21]. The cellular antioxidant enzymes have been found to be compromised in animal models and patients with OA [20,22–26]. An imbalance between oxidants and antioxidants results in oxidative damage, endoplasmic reticulum stress and mitochondrial dysfunction in chondrocytes (intrinsic pathway of apoptosis) [8,27–29], which subsequently leads to chondrocytic differentiation or apoptosis [30]. In addition the overexpression of death receptor 5 and TNF-related apoptosis-inducing ligand in the cartilage of OA patients [31], Fas-induced apoptosis of chondrocytes [6,32] may also contribute to the pathogenesis of OA.

The cartilage layer is avascular and alymphatic, with limited blood and oxygen supplies [33–35]. Chondrocytes adapt to this hypoxic condition via the constitutively activated 5′ adenosine monophosphate-activated protein kinase/Sirtuin-1 (AMPK/SIRT-1) signalling pathway [34,36–38]. AMPK signalling is important for energy production and regulation [36]. Recent evidence showed that SIRT-1 activation protects chondrocytes from apoptosis and radiation-induced senescence by improving mitochondrial function [39–41]. Both AMPK and SIRT-1 have been reported to inhibit inflammation and cartilage catabolism [39–42]. Studies showed that resveratrol (a SIRT-1 activator) can protect chondrocytes from oxidative stress, inflammation and apoptosis [43–46]. Furthermore, chondrocytes are highly dependent on autophagy as a reparatory mechanism during cellular damage, due to their limited mitotic capacity [33,47]. Autophagy removes any damaged or dysfunctional organelles, without compromising the cartilage cellularity [36,48]. Previous studies have demonstrated that autophagy processes and AMPK/SIRT-1 activities were compromised, via experimental models and patients with OA [48–50]. Conversely, AMPK and SIRT-1 activation could improve the progression of OA via autophagy induction [36,51,52]. Rapamycin (an autophagy inducer) and glucosamine (a joint supplement) were demonstrated to improve clinical signs of OA in experimental animals, by promoting autophagy of chondrocytes [53,54]. Glucosamine sulphate also significantly improves pain and joint function in the knees of OA patients [55–57], but its effect against joint space narrowing is moderate [35,58,59].

Circulating markers of cartilage catabolism can be used to monitor the progression of OA [60–63]. These markers include enzymes that are involved in matrix degradation in response to chondrocyte apoptosis, such as collagenases (matrix metalloproteinase (MMP)-1, -3, -8, and -13), gelatinases (MMP-2 and MMP-9) and aggrecanase [22,64–66]. The proinflammatory cytokines, such as TNF-α and IL-1β, upregulate these MMPs through signal transduction pathways [37,67]. In addition, ROS also cleave collagen and aggrecan in the cartilage matrix directly, via oxidation, nitrosylation, nitration or chlorination [6,22,30,64]. As a result, collagen and aggrecan levels in synovial fluid increase. These cartilage degradation products can further promote cartilage inflammation, chondrocyte apoptosis and ROS production, via a positive feedback loop [6,8,9]. Other cartilage proteins, such as lubricin and β-defensin-4, are also potential biomarkers for OA. Lubricin, or proteoglycan 4, is a type of glycoprotein that serves as a boundary lubricant between cartilage surfaces, temporomandibular joint discs and tendons [9,68,69]. Lubricin expression is lower in senescent or OA chondrocytes [69]. The inflammatory process in OA also upregulates β-defensin-4, an antimicrobial peptide, which can break down cartilage matrix by increasing MMP activity [70]. Higher levels of β-defensin-4 were found in chondrocytes isolated from OA patients in either monolayer culture or scaffold [70,71]. These pathways involved in chondrocyte apoptosis can be targeted for novel OA treatments [6].

In this context, olive oil and its active components (including its biologically-active polyphenols—hydroxytyrosol, tyrosol, oleocanthal and oleuropein) serve as potential candidates for the treatment of OA. Olive oil and its derivatives have demonstrated promising antioxidant and anti-inflammatory properties in isolated erythrocytes, in vitro cultured cells [72–78], exercise-exhausted rat skeletal muscle [79] and other animal disease models [80–84]. Furthermore, hydroxytyrosol was previously shown to improve mitochondrial respiration and reduce oxidative stress in the brain of db/db mice via AMPK activation [85]. Tyrosol and oleuropein were reported to decrease oxidative damage in cultured cells [86,87] and in a rat model of myocardial ischemia, by upregulating the expression of SIRT-1 and its nuclear translocation [88]. A recent study also reported that daily extra-virgin olive oil (EVOO) intake for six months improved synaptic integrity, with lower insoluble protein aggregation, via autophagy activation, in a rat model of Alzheimer's disease [89]. On the other hand, diets based on olive oil have reduced postprandial oxidative stress and inflammation in human studies [90–92]. These findings suggest that olive oil and its derivatives are potential therapeutic agents for inflammatory diseases like OA.

This review aimed to summarise the current evidence on the effects of olive oil and its derivatives on OA. A literature search was performed by the authors within the period of 1–31 July 2017 using the keywords 'olive OR tyrosol OR hydroxytyrosol OR oleocanthal OR oleuropein' AND 'osteoarthritis OR cartilage OR chondrocyte' in Pubmed and Scopus. Original research articles on human clinical trials, animals and cell culture studies, published in English, from the inception of the databases to the last date of the literature search (31 July 2017) were included. Studies on rheumatoid arthritis were excluded from this review. Fourteen studies were included for the final analysis.

2. Effects of Olive and Its Derivatives in Animal Models of OA

There are various methods to induce OA in experimental animals, such as injection of chemicals to induce collagen degradation or apoptosis of chondrocytes, surgical manoeuvres to destabilize the joint, the use of animals that have developed spontaneous OA and genetically modified animals. The animals of choice range from rodents to rabbits, dogs and monkeys. Each OA model and animal species has its pros and cons [93]. The studies included in this review used rodents and rabbits. OA developed spontaneously, or was induced using surgical methods. Progression of the disease was determined using histological assessments based on standard guidelines, collagen or glycoprotein expression in the cartilage layer, or serum biomarkers of cartilage degradation.

Gong et al. supplemented water extract of olive leaf in drinking water (500 mg/kg/day) in 16-week old New Zealand rabbits, through surgically-drilled holes at their stifle joints, for three weeks [94]. The surgery caused an invasion of macrophages and neutrophils in the cartilage layer of untreated rabbits, indicating an inflammatory process [94]. The subchondral bone also showed proliferation of osteoclasts, the bone-resorbing cells, indicating increased bone remodelling and subsequent bone damage [94]. Histological analyses showed a greater degree of healing in the supplemented rabbits relative to the untreated group. Proliferating chondrocytes, matured cartilage tissue and proteoglycans were present at the injured sites of supplemented rabbits [94]. The proliferation of osteoclasts at the subchondral bone was averted by using water extract of olive [94]. The researchers did not characterize the compounds found in the water extract used in this study, although the presence of hydroxytyrosol was suggested. The olive leaf extract was postulated to be anti-inflammatory, but pro-inflammatory cytokine levels were not examined. Nevertheless, this study provided clues that olive leaf extract possesses joint-protective properties.

Extra-virgin olive oil (EVOO), commonly consumed in Mediterranean diets, contains a high amount of polyphenols (for example, oleuropein, tyrosol and hydroxytyrsosol) and unsaturated fatty acids (for example: oleic and linoleic fatty acid), because its production does not involve thermal and physical alternation of the oil [95,96]. Musumeci et al. compared the effects of mild physical activities (treadmill training), an EVOO-enriched diet, and the combination of both, in a rat model of OA, which was induced through anterior cruciate ligament transection for eight weeks [97].

The fat component of the experimental diet (30% of the total weight of the diet) was replaced with EVOO [97]. The combination of treadmill training and the EVOO-enriched diet prevented cartilage damage, as assessed using Kraus' modified Mankin score and the Osteoarthritis Research Society International (OARSI) histopathology scoring system [97]. This was probably achieved through enhanced expression of lubricin, which is a mucinous glycoprotein, which coats the surface of cartilage to reduce friction [98]. The synovial lubricin level decreased in rats induced with OA early during the joint's acute inflammation phase [97]. The rats receiving combination therapy showed an increase in synovial lubricin levels, concurrently with a reduction in interleukin-1 levels [97]. Individual treatments were less effective in preventing cartilage damage and increasing lubricin expression, indicating a synergistic effect between exercise and EVOO-enriched diets [97]. The content of EVOO used was not scrutinized in this study.

Olive extract was also mixed with other compounds to explore their synergistic joint protective effects. Mével et al. supplemented olive and grape seed extracts in 10-week-old male C57/BL mice (4 g/kg/day) for four weeks, and 15-week-old female New Zealand white rabbits (100 mg/kg/day) for three weeks, before destabilizing their joints surgically (bilateral destabilization of the medial meniscus for mice; anterior cruciate ligament transection of the right joint for rabbits) [99]. The supplementation, which was standardized according to hydroxytyrosol (bioactive compound in olive) and procyanidins (bioactive compound in grape seed), continued for eight weeks for mice and ten weeks for rabbits [99]. Both mice and rabbits receiving treatment showed decreased OARSI scores and cartilage abrasion at the knee, relative to their negative controls [99]. In comparison, cartilage erosion to the mid-zone layer was observed in animals fed with glucosamine hydrochloride, indicating that olive and grape seed extracts showed better chondroprotective effects [99]. The decrease in aggrecan expression in the cartilage layer due to OA was prevented by the treatment [99].

Oleuropein, another major polyphenol in olives, was also investigated for its chondroprotective effects. Horcajada et al. compared the effects of a diet enriched with oleuropein (0.025% or 12.5 mg/kg body weight), rutin (a glycoside found in citrus fruit; 0.5% or 50 mg/kg body weight) or rutin and curcumin together (principal curcuminoid found in turmeric; 0.5%/0.25% or 50 mg/kg rutin and 125 mg/kg body weight curcumin) on the joints of four-week-old Dunkin–Hartley guinea pigs, for 31 weeks [100]. These animals developed OA spontaneously at 35-weeks of age [100]. The supplemented groups experienced varying degrees of improvement in global OA histological scores at the joints. Joint lesions of the groups supplemented with oleuropein and rutin at the femoral, tibial, medial and lateral compartments were less severe. Cellularity scores were lower in the oleuropein and rutin plus curcumin groups. Osteophyte scores were lower in the oleuropein group. Synovial scores were improved by oleuropein and rutin, and to a lesser degree, in the rutin plus curcumin supplemented group [100]. Changes in serum biochemical markers of OA are correlated with OA progression, whereby a higher level usually indicates elevated inflammation or cartilage degradation. In this study, all treatments could not suppress the serum nitrated collagen levels (cartilage degradation marker) due to OA. Oleuropein supplementation reduced the level of PGE_2 (inflammation marker) and collagen-2 (cartilage degradation marker). Rutin alone, or in combination with curcumin, decreased collagen-2 and aggrecan levels (cartilage degradation marker) but did not affect PGE_2 levels. Rutin, in combination with cucurmin, reduced fibulin-3 fragment levels (cartilage degradation marker) [100]. Overall, oleuropein and rutin individually showed potential anti-osteoarthritic effects, but synergistic effects were not seen in the rutin plus curcumin group.

In summary, olive extract, EVOO and polyphenols derived from olive trees possess potent chondroprotective effects, for example decreasing cartilage lesions and degradation in various animal models of OA. The underlying mechanisms could be due to decreased inflammation and enhanced lubricin expression. Cartilage regeneration by olive and its derivatives at the late stage of OA has not been studied so far. Late stage OA often requires surgical intervention, and physical rehabilitation and exercise have been recommended to help patients to regain function [101]. As shown by Musumeci et al., EVOO and physical activity act synergistically in preventing OA in rats [97]. Olive and

its derivatives could be administered along with physical activity in rehabilitation programmes, to help OA patients regenerate cartilage and regain function [35]. This speculation should be validated in an animal study.

3. Effects of Olive and Its Derivatives in Human Studies

There have been four reports on the efficacy of olive and its derivatives on OA patients, of which three were randomized controlled trials [102–104] and one was a small-scale uncontrolled trial [105]. Subjects included in these studies were patients with OA [102–105]. An intervention, in the form of topical (olive extract and virgin olive oil) [103,105] or oral supplementation (olive extract and hydroxytyrosol) [102,104] was given. Comparison with a placebo [102,104] or an analgesic (piroxicam) [103] was performed in three of the clinical trials. Outcomes were measured using standardized OA questionnaires [102–104].

In a preliminary study, five subjects with symptomatic OA (aged 60.2 ± 8.1 years) applied 5 g of ointment, containing a 5% unsaponifiable fraction of unripe olive fruits, to their painful knee and hand joints, three times a day, for two–three weeks [105]. Weekly assessment of pain was performed using a visual analogue scale. Inflammation was inspected visually by physicians [105]. The unsaponifiable fraction contained lanosterol (2.60 mg/g oil), stigmasterol (2.15 mg/g oil), cycloartanol acetate (2.04 mg/g oil), stigmastan-3,5-diene (2.01 mg/g oil), obtusifoliol (1.93 mg/g oil), cholesta-4,6-dien-3-one (1.42 mg/g oil), α-amyrin (1.42 mg/g oil), α-tocopherol (1.32 mg/g oil), squalene (1.02 mg/g oil), β-amyrin (0.57 mg/g oil), and β-sitosterol (0.22 mg/g oil) [105]. The subjects experienced less joint pain and oedema, and improved mobility, one week after initiation of the treatment [105]. Redness and heat improved two weeks after the treatment. No adverse reactions were reported [105]. This pilot study provided some early evidence for the effects of olive derivatives on joint pain. However, it was a time-series study, without proper blinding, controls or randomization. The duration of treatment was relatively short (two–three weeks).

Following the aforementioned study, a double-blinded randomized clinical trial was performed on female Iranian osteoarthritic patients, aged 40–85 years [103]. The treatment group (n = 30) applied virgin olive oil topically, while the control group (n = 30) applied 1 g of piroxicam (NSAID) gel (0.5%) three times daily, for four weeks [103]. Both topical piroxicam and olive oil decreased Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) pain subscale scores and secondary outcome measures of the subjects [103]. The performance of olive oil was superior to piroxicam, starting at week two after initiation [103]. Only one patient complained of skin allergy after the olive oil application [103]. The dropout rate of this study was high, but the rate between treatment and controls was not significantly different. This might have affected the power of the study. In addition, the duration of treatment was relatively short [103].

Apart from topical application, the efficacy of olive extract supplement has also been studied. The effect of oral olive extract supplement in patients with OA (aged 55–75 years) was first tested by Bitler et al. in a randomized double-blinded placebo-controlled trial [102]. The treatment group (n = 30) took 400 mg of freeze-dried olive water extract orally for eight weeks. Osteoarthritic patients in the treatment arm showed significant improvements, as indicated by the Health Assessment Questionnaire-Disability Index, Disease Activity Score with 28-Joint Count Index [102].

Another randomized double-blinded placebo-controlled clinical trial tested the effects of oral hydroxytyrosol supplementation on knee OA. Subjects with knee pain were treated with 50.1 mg/day olive extract containing 10.04 mg hydroxytyrosol for four weeks (n = 13; aged 60.8 ± 7.2 years) [104]. They showed a higher improvement, based on Japanese Orthopaedic Association Scores, compared to the placebo group (total score) [104]. Scores for pain during sleeping at night were significantly reduced in the treatment group. Scores for pain during walking on a flat plane were improved marginally as well. Other types of pain were not improved significantly by hydroxytyrosol treatment [104]. In both oral supplementation studies, the number of participants was low and the duration of action was short [104].

A summary of the evidence from human clinical trials on the efficacy of olive and its derivatives is presented in Table 1. All the studies reviewed were small in sample size and short in duration. They used scoring systems to evaluate improvements in the subjects. More objective measurements, like joint space evaluation using X-ray images, could help to validate the efficacy of olive derivatives in improving OA.

Table 1. A summary of the findings from human studies on the efficacy of olive and its derivatives against osteoarthritis.

Authors	Study Design	Patients, Interventions, Comparisons	Outcomes
Bitler et al. 2007 [102]	Randomized, double-blinded, placebo-controlled trial.	Patients with OA or RA, aged 55 to 75 years, free from other chronic diseases. Treatment group: 13 RAs and 30 OAs; 400 mg of freeze-dried olive water extract per day for 8 weeks Placebo group: 14 RAs and 33 OAs	OA patients receiving treatment showed significant improvements, as indicated by the Health Assessment Questionnaire–Disability Index, Disease Activity Score With 28-Joint Count index.
Bhoololi et al. 2012 [103]	Randomized, standard-controlled trial.	Female participants from a clinic in Iran, aged between 40–85 years diagnosed with OA. Treatment: 30 patients, virgin olive oil Control: 30 patients, 0.5% piroxicam 1 g gel, 3 times daily, for 4 weeks.	Both topical piroxicam and olive oil decreased WOMAC pain subscale scores and secondary outcome measures for the subjects. The performance of olive oil was superior compared to piroxicam, starting at week 2. Only one patient suffered a skin allergy after olive oil application.
Takeda et al. 2013 [104]	Double-blinded placebo-controlled trial.	Men and women with knee pain (gonarthrosis) Treatment group (13): aged 60.8 ± 7.2 years; 50.1 mg/day olive extract containing 10.04 mg hydroxytyrosol for 4 weeks. Placebo group (12): aged 61.4 ± 8.3 years.	Total improvement, based on JOA scores, was higher in the treated group compared to the placebo group, but not for subscales. Pain scores for pain during sleeping at night was significantly reduced for the treated group compared to the placebo group, pain during walking in flat planes was marginally signficiant, but other reductions in pain were not signficant.
Gelmini et al. 2015 [105]	Uncontrolled trial.	5 humans (men and women), 60.2 ± 8.1 years, diagnosed with symptomatic OA. They applied 5 g of oinment to their painful joints on knee and hands three times a day for 2–3 weeks. The oinment contained a 5% unsaponifiable fraction from unripe olive oil.	Joint pain, oedema and mobility started to improve after week 1. Redness and heat started to improve after week 2. No adverse reactions were reported.

Abbreviation: JOA = Japanese Osteoporosis Association; RA = rheumatoid arthritis; OA = osteoarthritis; WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index.

4. Molecular Effects of Olive and Its Derivatives in Cartilage Protection

Polyphenols from olive leaves and EVOO are postulated to exert their chondroprotective effects via anti-inflammatory actions. Nsir et al. pre-treated chondrocytes derived from OA patients and challenged with lipopolysaccharide (LPS, an inflammation inducer) with olive leaves and EVOO extracts of different polarities and maturities [106]. A chemical analysis showed that extract from olive leaves showed the highest degree of reducing power compared to various EVOOs [106]. All extracts did not induce cytotoxicity in the chondrocytes, and did not potentiate the effects of LPS [106]. Of note, EVOO derived from unripe olives displayed superior inflammatory activity, by abolishing the protein expression of iNOS, while the other extracts only reduced its expression [106]. Apolar fractions of EVOO could preserve the function of chondrocytes by maintaining their ability to produce collagen type-2, despite being challenged with LPS [106]. This may be due to the presence of lipid soluble substances, such as alpha-tocopherol and fatty acids, in the apolar fraction of EVOO.

As an extension of their animal studies, Mével et al. treated rabbit chondrocytes with olive and grape seed extracts before exposing them to IL-1β [99]. The extract reduced the expression of iNOS, NO, cyclooxygenase-2 (COX-2) and PGE_2 involved in inflammation, as well as cartilage degradation markers (MMP-13) [99]. The chondrocytes were also treated with sera of rabbits, fed with the extract at a dose of 500 mg/kg for eight days, to demonstrate that metabolites from the extract could be responsible for the observed chondroprotective effects [99]. The experiment showed that the sera prevented increases in NO, PGE_2 and MMP13 due to interleukin-1 β stimulation [99].

Oleocanthal is an olive polyphenol which has gained considerable attention because it exerts effects similar to non-steroidal anti-inflammatory agents. Pre-treatment of ATDC-5 chondrocytes with oleocanthal and its derivatives protected chondrocytes from LPS-induced cell death at low concentrations (1–10 μM) [107]. This was achieved by reducing the expression of iNOS and subsequently the production of NO [107]. The actions of oleocanthal were accompanied by phosphorylation of p38 (which contributes either to cell survival or apoptosis) [107]. In contrast, this side effect was not seen with some oleocanthal derivatives [107]. The researchers indicated that the lack of p38 activation was beneficial, because it could cause apoptosis of other cells in the body. However, further study on additional pathways that govern the direction of p38 activation (survival/apoptosis) was not performed. The protein expression of inflammatory cytokines, such as IL-1β, TNF-α and granulocyte macrophage colony-stimulating factor in chondrocytes was also reduced by oleocanthal [108]. The effects of oleocanthal were also tested on macrophages since they are an important source of inflammatory cytokines [108]. Oleocanthal was found to reduce NO production by the murine macrophage, J774A.1, which is stimulated with LPS [108]. This was achieved by suppressing the protein expression of nitrite oxide synthase type-2 [108]. Oleocanthal also decreased the protein and gene expressions of macrophage inflammatory protein 1α and interleukin-6, in both macrophages and ATDC-5 chondrocytes [108].

Another olive polyphenol, hydroxytyrosol, is a potent antioxidant capable of modulating specific signalling pathways in chondrocytes. Previous reports showed that hydroxytyrosol reduced radical oxygen species production and associated DNA breakage induced by H_2O_2, in human primary chondrocytes [109]. It also prevented apoptosis of chondrocytes, induced by H_2O_2, by suppressing the increase in caspase-3 [109]. Increased inflammation (indicated by elevated COX-2 and iNOS), cartilage degradation (indicated by MMP-13), and terminal chondrocytic differentiation (indicated by runt-related transcription factor 2 (RUNX-2)) and vascular endothelial growth factor (VEGF) (induced by H_2O_2 and growth-related oncogene α (GROα)) were prevented by hydroxytyrosol [109]. mRNA expression of SIRT-1, an antiaging gene associated with OA, was lowered by GROα, but this was reversed with hydroxytyrosol treatment [109]. Later experiments showed that hydroxytyrosol increases the expression of SIRT-1 in primary human chondrocytes and C-28/I2 chondrocytes, with or without the presence of H_2O_2 [110]. This effect was modulated partially by microRNA-9 (miR-9), since pre-miR-9 transfection in chondrocytes partially reverses the beneficial effects of hydroxytyrosol in reducing cell death and caspase-3 when challenged with H_2O_2 [110]. The transfected cells also expressed higher levels of MMP-13, VEGF and RUNX-2, but their levels in cells treated with hydroxytyrosol remained low, compared to untreated cells [110].

Impaired autophagy has been associated with the pathogenesis of OA [111]. Autophagy removes impaired organelles and macromolecules in the chondrocytes to ensure they are healthy. Autophagy increases in chondrocytes during stress (early OA), but decreased autophagy is linked to cell death (late OA) [112]. Hydroxytyrosol could elevate protein expression of the autophagy marker, microtubule-associated protein 1A/1B-light chain 3 conjugate (LC3-II), and decrease the expression of p62 in chondrocytes, with or without the presence of H_2O_2 [113]. LC3-II is involved in the formation of the autophagy membrane, whereas p62 is a substrate used during autophagy [114]. Fluorescent staining also demonstrated an increase in late autophagy compartments (autolysosomes) in hydroxytyrosol-treated cells [113]. The addition of autophagy inhibitors completely abolished the protective effects of hydroxytyrosol on chondrocytes when challenged

with H_2O_2 [113]. This effect was modulated by SIRT-1, because silencing of SIRT-1 abolished the antiapoptotic effects of hydroxytyrosol when chondrocytes were challenged with H_2O_2, and upregulated LC3-II expression [113]. However, silencing of SIRT-1 did not affect p62 expression [113]. Hydroxytyrosol influenced SIRT-1 by increasing its movement into the nucleus [113]. It was postulated that SIRT-1 in the nucleus catalysed the deacylation of LC3-II, which then crossed into the cytosol to initiate autophagy [113].

In summary, olive oleocanthals possess anti-inflammatory activity by suppressing iNOS and NO production. Previous studies have shown that olive polyphenols, like hydroxytyrosol, can prevent degradation of the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) and prevent nuclear translocation of p65, thereby suppressing inflammation governed by the NFκB pathway [115,116]. Hydroxytyrosol also exerts more specific actions, such as reducing SIRT-1 expression and maintaining autophagy processes in chondrocytes and cell homeostasis, thereby preventing apoptosis of chondrocytes when challenged with oxidative or inflammatory assaults. These mechanisms prevent the breakdown of the cartilage layer and slow down the progression of OA (Figure 1). How far these in vitro processes mimic in vivo conditions, is debatable. Chondrocytes might behave differently when cultured in monolayer. Of all the cell culture studies summarized, only one grew the chondrocytes on a scaffold [109]. Only one study addressed the possibility that the metabolites, instead of the parent compounds derived from olive, exerted chondroprotective effects [99]. These are some of the issues to be resolved in the future.

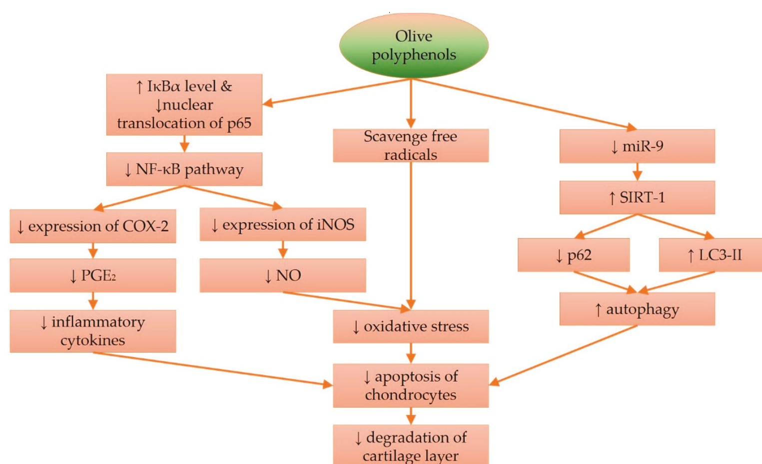


Figure 1. The mechanisms of action of olive polyphenols in preventing osteoarthritis. Abbreviations: COX-2 = cyclooxygenase-2; IκBα = nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; iNOS = inducible nitric oxide synthase; miR= microRNA; NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells; LC3-II = microtubule-associated protein 1A/1B-light chain 3 conjugate, p62 = nucleoporin p62; p65 = transcription factor p65; PGE_2 = prostaglandin E_2; SIRT-1 = sirtuin-1.

5. Perspectives on the Use of Olive and Its Derivatives in Combating OA

Pharmacokinetic studies have shown that polyphenols from virgin olive oil can be absorbed in realistic doses by humans [117]. The intestinal absorption of olive polyphenols, as represented by tyrosol and hydroxytyrosol levels, is around 55–56% in humans [118]. Oleuropein is metabolised and excreted as hydroxytyrosol [118]. The oral bioavailability of tyrosol and hydroxytyrosol is higher in olive oil compared to aqueous solutions [119]. Hydroxytyrosol undergoes extensive metabolism, mainly through glucoronidation processes in the liver [120]. Plasma concentrations of hydroxytyrosol

peak at about 30 min and decrease henceforth [120]. The elimination half-life of hydroxytyrosol is 2.45 h [120]. The glucuronide conjugates of tyrosol and hydroxytyrosol are excreted in urine [121]. Olive polyphenols, or their metabolites, must be distributed to the respective body tissues to exert their biological effects. Since the cartilage layer is avascular, chondrocytes obtain nutrients from the synovial fluid. Data on the distribution of olive polyphenols in synovial fluid are limited. This is probably due to the invasiveness of the procedure involved, and the small volume of synovial fluid in animals.

Parkinson and Cicerale estimated that the average intake of olive polyphenols is 200 μg/day for humans practising traditional Mediterranean diets, assuming that 30–50 g of olive oil is consumed daily [122]. This is equivalent to a dose of 17.6 μg/kg body weight for rats or 10.3 μg/kg body weight for rabbits, based on the body surface ratio (km of a human with a body weight of 60 kg = 37; km of a large rat = 7; km of a rabbit = 12) [123]. In comparison, animal studies summarized in this review that demonstrated beneficial effects of olive polyphenols used much larger doses [94,99]. The study by Musumeci et al., which replaced the fat content of a standard diet with EVOO, was more similar to human consumption patterns. However, they failed to show beneficial effects in the group treated with EVOO alone [97]. This prompts the question whether the polyphenol content in normal olive oil consumption is sufficient to exert any chondroprotective effects, and whether supplementation at higher doses is required.

Nevertheless, there are ways to improve the absorption of olive polyphenols using innovative delivery systems, through oral or topical routes. Ng et al. showed that fresh freeze-dried olive (containing around 3% hydroxytyrosol), delivered topically via an arginine bilayer film, could reduce arthritic scores, histological scores, paw and ankle circumferences and circulating interleukin-6 in a rat model of rheumatoid arthritis, induced by Freund's adjuvant [124]. A topical formulation of hydrocortisone–hydroxytyrosol loaded chitosan nanoparticles has been developed to treat dermatitis [125]. Hydroxytyrosol loaded in an emulsion system could be delivered orally to enhance its bioavailability [126]. A gelled double emulsion system could reduce the degradation of hydroxytyrosol due to gastric acid and digestive enzymes [127].

Recent studies also highlight the role of adiponetins in the pathogenesis of OA [128–130]. Adipokines, including leptin, adiponectin and resistin, are expressed in adipose tissues, osteoclasts, osteoblasts and chondrocytes [128,131]. The exact role of these adipokines in OA is controversial. Leptin is higher in the synovial fluid of obese and OA patients [129,132]. Leptin was demonstrated to induce cartilage metabolism and inflammation via the induction of IL-1β, NO, PGE_2, MMP-9 and MMP-13 expression [133,134]. However, leptin was also shown to increase chondrocyte proliferation, which contradicted its role as a proinflammatory molecule [129]. On the other hand, adiponectin levels are decreased in obese and diabetic patients [135]. This has been shown to downregulate IL-1β-induced MMP-13 expression and upregulate tissue inhibitors of MMP-2, to protect against OA [128,136]. However, adiponectin was also reported to induce the expression of iNOS and the release of IL-6, MMP-3 and MMP-9 from chondrocytes [128,137–139]. Diets based on olive oil were shown to increase adiponectin production by the adipocytes, and circulating adiponectin levels in overweight women. Thus, olive derivatives could influence the pathogenesis of OA via adiponectin, but this awaits validation [140,141].

This review is not without its limitations. Firstly, it only focuses on the anti-osteoarthritis effects of olive oil and its polyphenols. However, other compounds present in olive oil, such as alpha-tocopherol (the predominant form of vitamin E in nature), oleic and linoleic acids (both are the predominant unsaturated fatty acids in olive oil) also possess antioxidant and anti-inflammatory effects, which might contribute to the anti-osteoarthritic properties of olive oil [142,143]. All these compounds could act synergistically to provide better chondroprotective effects than olive polyphenols alone. The interaction of olive polyphenols with other food components, especially those found in Mediterranean diets, should also be studied, because of similar synergistic effects. Some studies summarised in this review have shown that procyanidins found in grape seeds might enhance the beneficial effects of olive polyphenols on cartilage [99]. Secondly, OA was involved not only in the degeneration of cartilage,

but also in changes in subchondral bone, osteophyte formation, inflammation of synovium tissues and tendons, as well as muscle weakness [144]. Most of the mechanistic studies here focus on chondrocytes per se. Olive and its polyphenols are effective in preventing bone loss due to sex hormone deficiencies and chronic inflammation [145]. Therefore, they may also prevent destruction of subchondral bone during early OA [144]. Improving subchondral bone integrity has been proven to reduce the severity of OA [146]. There are limited human studies on the effects of olive and its derivatives on OA, and the available literature from the search has been included in this review. This highlighted the need for more well-planned human clinical trials to validate the role of olive and its derivatives in preventing OA.

6. Conclusions

Olive and its derivatives show potential in preventing cartilage damage due to OA. This is attributed to their antioxidant and anti-inflammatory effects. In particular, hydroxytyrosol can modulate the SIRT-1 gene to improve autophagy and survival of chondrocytes. This review suggests that olive and its derivatives by themselves, or in combination with other approaches like physical activity, could be used to retard the progression of OA in individuals at risk. Current human studies suggest some improvements in the functional and pain scores in OA patients treated with olive extract, topically or orally, but more evidence from well-planned clinical trials to support the use of olive supplements in OA patients is required. Furthermore, the role of olive and its derivatives in late stage OA and post-surgical rehabilitation of OA patients require more research.

Acknowledgments: We thank Universiti Kebangsaan Malaysia for supporting the research via FF-2016-119 and FF-2017-270.

Author Contributions: Kok-Yong Chin conceived the idea and performed the literature search. Kok-Yong Chin and Kok-Lun Pang wrote the manuscript. Kok-Yong Chin gave the final approval for publishing the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kraus, V.B.; Blanco, F.J.; Englund, M.; Karsdal, M.A.; Lohmander, L.S. Call for standardized definitions of osteoarthritis and risk stratification for clinical trials and clinical use. *Osteoarthr. Cartil.* **2015**, *23*, 1233–1241. [CrossRef] [PubMed]
2. Centers for Disease Control and Prevention. Osteoarthritis (OA). Available online: http://www.cdc.gov/arthritis/basics/osteoarthritis.htm (accessed on 18 June 2016).
3. Cross, M.; Smith, E.; Hoy, D.; Nolte, S.; Ackerman, I.; Fransen, M.; Bridgett, L.; Williams, S.; Guillemin, F.; Hill, C.L.; et al. The global burden of hip and knee osteoarthritis: Estimates from the global burden of disease 2010 study. *Ann. Rheum. Dis.* **2014**, *73*, 1323–1330. [CrossRef] [PubMed]
4. Salmon, J.H.; Rat, A.C.; Sellam, J.; Michel, M.; Eschard, J.P.; Guillemin, F.; Jolly, D.; Fautrel, B. Economic impact of lower-limb osteoarthritis worldwide: A systematic review of cost-of-illness studies. *Osteoarthr. Cartil.* **2016**, *24*, 1500–1508. [CrossRef] [PubMed]
5. Musumeci, G.; Szychlinska, M.A.; Mobasheri, A. Age-related degeneration of articular cartilage in the pathogenesis of osteoarthritis: Molecular markers of senescent chondrocytes. *Histol. Histopathol.* **2015**, *30*, 1–12. [PubMed]
6. Mobasheri, A.; Matta, C.; Zakany, R.; Musumeci, G. Chondrosenescence: Definition, hallmarks and potential role in the pathogenesis of osteoarthritis. *Maturitas* **2015**, *80*, 237–244. [CrossRef] [PubMed]
7. Cheng, N.T.; Meng, H.; Ma, L.F.; Zhang, L.; Yu, H.M.; Wang, Z.Z.; Guo, A. Role of autophagy in the progression of osteoarthritis: The autophagy inhibitor, 3-methyladenine, aggravates the severity of experimental osteoarthritis. *Int. J. Mol. Med.* **2017**, *39*, 1224–1232. [CrossRef] [PubMed]
8. Musumeci, G.; Castrogiovanni, P.; Trovato, F.M.; Weinberg, A.M.; Al-Wasiyah, M.K.; Alqahtani, M.H.; Mobasheri, A. Biomarkers of chondrocyte apoptosis and autophagy in osteoarthritis. *Int. J. Mol. Sci.* **2015**, *16*, 20560–20575. [CrossRef] [PubMed]

9. Musumeci, G.; Aiello, F.C.; Szychlinska, M.A.; Di Rosa, M.; Castrogiovanni, P.; Mobasheri, A. Osteoarthritis in the XXist century: Risk factors and behaviours that influence disease onset and progression. *Int. J. Mol. Sci.* **2015**, *16*, 6093–6112. [CrossRef] [PubMed]
10. Musumeci, G.; Castrogiovanni, P.; Loreto, C.; Castorina, S.; Pichler, K.; Weinberg, A.M. Post-traumatic caspase-3 expression in the adjacent areas of growth plate injury site: A morphological study. *Int. J. Mol. Sci.* **2013**, *14*, 15767–15784. [CrossRef] [PubMed]
11. Nakki, A.; Rodriguez-Fontenla, C.; Gonzalez, A.; Harilainen, A.; Leino-Arjas, P.; Heliovaara, M.; Eriksson, J.G.; Tallroth, K.; Videman, T.; Kaprio, J.; et al. Association study of MMP8 gene in osteoarthritis. *Connect. Tissue Res.* **2016**, *57*, 44–52. [CrossRef] [PubMed]
12. Pozgan, U.; Caglic, D.; Rozman, B.; Nagase, H.; Turk, V.; Turk, B. Expression and activity profiling of selected cysteine cathepsins and matrix metalloproteinases in synovial fluids from patients with rheumatoid arthritis and osteoarthritis. *Biol. Chem.* **2010**, *391*, 571–579. [CrossRef] [PubMed]
13. Scanzello, C.R.; Umoh, E.; Pessler, F.; Diaz-Torne, C.; Miles, T.; Dicarlo, E.; Potter, H.G.; Mandl, L.; Marx, R.; Rodeo, S.; et al. Local cytokine profiles in knee osteoarthritis: Elevated synovial fluid interleukin-15 differentiates early from end-stage disease. *Osteoarthr. Cartil.* **2009**, *17*, 1040–1048. [CrossRef] [PubMed]
14. Janusz, M.J.; Little, C.B.; King, L.E.; Hookfin, E.B.; Brown, K.K.; Heitmeyer, S.A.; Caterson, B.; Poole, A.R.; Taiwo, Y.O. Detection of aggrecanase- and MMP-generated catabolic neoepitopes in the rat iodoacetate model of cartilage degeneration. *Osteoarthr. Cartil.* **2004**, *12*, 720–728. [CrossRef] [PubMed]
15. Fuchs, S.; Skwara, A.; Bloch, M.; Dankbar, B. Differential induction and regulation of matrix metalloproteinases in osteoarthritic tissue and fluid synovial fibroblasts. *Osteoarthr. Cartil.* **2004**, *12*, 409–418. [CrossRef] [PubMed]
16. Sakurai, H.; Kohsaka, H.; Liu, M.F.; Higashiyama, H.; Hirata, Y.; Kanno, K.; Saito, I.; Miyasaka, N. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. *J. Clin. Investig.* **1995**, *96*, 2357–2363. [CrossRef] [PubMed]
17. Hiran, T.S.; Moulton, P.J.; Hancock, J.T. Detection of superoxide and NADPH oxidase in porcine articular chondrocytes. *Free Radic. Biol. Med.* **1997**, *23*, 736–743. [CrossRef]
18. Rathakrishnan, C.; Tiku, K.; Raghavan, A.; Tiku, M.L. Release of oxygen radicals by articular chondrocytes: A study of luminol-dependent chemiluminescence and hydrogen peroxide secretion. *J. Bone Miner. Res.* **1992**, *7*, 1139–1148. [CrossRef] [PubMed]
19. Khan, I.M.; Gilbert, S.J.; Caterson, B.; Sandell, L.J.; Archer, C.W. Oxidative stress induces expression of osteoarthritis markers procollagen IIA and 3B3(-) in adult bovine articular cartilage. *Osteoarthr. Cartil.* **2008**, *16*, 698–707. [CrossRef] [PubMed]
20. Ostalowska, A.; Birkner, E.; Wiecha, M.; Kasperczyk, S.; Kasperczyk, A.; Kapolka, D.; Zon-Giebel, A. Lipid peroxidation and antioxidant enzymes in synovial fluid of patients with primary and secondary osteoarthritis of the knee joint. *Osteoarthr. Cartil.* **2006**, *14*, 139–145. [CrossRef] [PubMed]
21. Tiku, M.L.; Liesch, J.B.; Robertson, F.M. Production of hydrogen peroxide by rabbit articular chondrocytes. Enhancement by cytokines. *J. Immunol.* **1990**, *1990*, 690–696.
22. Scott, J.L.; Gabrielides, C.; Davidson, R.K.; Swingler, T.E.; Clark, I.M.; Wallis, G.A.; Boot-Handford, R.P.; Kirkwood, T.B.; Taylor, R.W.; Young, D.A. Superoxide dismutase downregulation in osteoarthritis progression and end-stage disease. *Ann. Rheum. Dis.* **2010**, *69*, 1502–1510. [CrossRef] [PubMed]
23. Regan, E.; Flannelly, J.; Bowler, R.; Tran, K.; Nicks, M.; Carbone, B.D.; Glueck, D.; Heijnen, H.; Mason, R.; Crapo, J. Extracellular superoxide dismutase and oxidant damage in osteoarthritis. *Arthritis Rheumatol.* **2005**, *52*, 3479–3491. [CrossRef] [PubMed]
24. Aigner, T.; Fundel, K.; Saas, J.; Gebhard, P.M.; Haag, J.; Weiss, T.; Zien, A.; Obermayr, F.; Zimmer, R.; Bartnik, E. Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. *Arthritis Rheumatol.* **2006**, *54*, 3533–3544. [CrossRef] [PubMed]
25. Ruiz-Romero, C.; Calamia, V.; Mateos, J.; Carreira, V.; Martinez-Gomariz, M.; Fernandez, M.; Blanco, F.J. Mitochondrial dysfunction of osteoarthritic human articular chondrocytes analyzed by proteomics. *Mol. Cell. Proteom.* **2009**, *8*, 172–189. [CrossRef] [PubMed]
26. Altay, M.A.; Erturk, C.; Bilge, A.; Yapti, M.; Levent, A.; Aksoy, N. Evaluation of prolidase activity and oxidative status in patients with knee osteoarthritis: Relationships with radiographic severity and clinical parameters. *Rheumatol. Int.* **2015**, *35*, 1725–1731. [CrossRef] [PubMed]

27. Maneiro, E.; Lopez-Armada, M.J.; de Andres, M.C.; Carames, B.; Martin, M.A.; Bonilla, A.; Del Hoyo, P.; Galdo, F.; Arenas, J.; Blanco, F.J. Effect of nitric oxide on mitochondrial respiratory activity of human articular chondrocytes. *Ann. Rheum. Dis.* **2005**, *64*, 388–395. [CrossRef] [PubMed]
28. Johnson, K.; Jung, A.; Murphy, A.; Andreyev, A.; Dykens, J.; Terkeltaub, R. Mitochondrial oxidative phosphorylation is a downstream regulator of nitric oxide effects on chondrocyte matrix synthesis and mineralization. *Arthritis Rheumatol.* **2000**, *43*, 1560–1570. [CrossRef]
29. Rachek, L.I.; Grishko, V.I.; Ledoux, S.P.; Wilson, G.L. Role of nitric oxide-induced mtDNA damage in mitochondrial dysfunction and apoptosis. *Free Radic. Biol. Med.* **2006**, *40*, 754–762. [CrossRef] [PubMed]
30. Henrotin, Y.E.; Bruckner, P.; Pujol, J.P.L. The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthr. Cartil.* **2003**, *11*, 747–755. [CrossRef]
31. Musumeci, G.; Loreto, C.; Carnazza, M.L.; Martinez, G. Characterization of apoptosis in articular cartilage derived from the knee joints of patients with osteoarthritis. *Knee Surg. Sports Traumatol. Arthrosc.* **2011**, *19*, 307–313. [CrossRef] [PubMed]
32. Hashimoto, S.; Satareh, M.; Ochs, R.L.; Lotz, M. Fas/fas ligand expression and induction of apoptosis in chondrocytes. *Arthritis Rheumatol.* **1997**, *40*, 1749–1755. [CrossRef]
33. Aigner, T.; Hemmel, M.; Neureiter, D.; Gebhard, P.M.; Zeiler, G.; Kirchner, T.; McKenna, L. Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritic human articular knee cartilage. *Arthritis Rheumatol.* **2001**, *44*, 1304–1312. [CrossRef]
34. Doerks, T.; Copley, R.R.; Schultz, J.; Ponting, C.P.; Bork, P. Systematic identification of novel protein domain families associated with nuclear functions. *Genome Res.* **2002**, *12*, 47–56. [CrossRef] [PubMed]
35. Castrogiovanni, P.; Trovato, F.M.; Loreto, C.; Nsir, H.; Szychlinska, M.A.; Musumeci, G. Nutraceutical supplements in the management and prevention of osteoarthritis. *Int. J. Mol. Sci.* **2016**, *17*, 2042. [CrossRef] [PubMed]
36. Bohensky, J.; Leshinsky, S.; Srinivas, V.; Shapiro, I.M. Chondrocyte autophagy is stimulated by HIF-1 dependent AMPK activation and mtor suppression. *Pediatr. Nephrol.* **2010**, *25*, 633–642. [CrossRef] [PubMed]
37. Terkeltaub, R.; Yang, B.; Lotz, M.; Liu-Bryan, R. Chondrocyte AMP-activated protein kinase activity suppresses matrix degradation responses to proinflammatory cytokines interleukin-1β and tumor necrosis factor α. *Arthritis Rheumatol.* **2011**, *63*, 1928–1937. [CrossRef] [PubMed]
38. Thoms, B.L.; Dudek, K.A.; Lafont, J.E.; Murphy, C.L. Hypoxia promotes the production and inhibits the destruction of human articular cartilage. *Arthritis Rheumatol.* **2013**, *65*, 1302–1312. [CrossRef] [PubMed]
39. Gabay, O.; Sanchez, C.; Dvir-Ginzberg, M.; Gagarina, V.; Zaal, K.J.; Song, Y.; He, X.H.; McBurney, M.W. Sirtuin 1 enzymatic activity is required for cartilage homeostasis in vivo in a mouse model. *Arthritis Rheumatol.* **2013**, *65*, 159–166. [CrossRef] [PubMed]
40. Matsushita, T.; Sasaki, H.; Takayama, K.; Ishida, K.; Matsumoto, T.; Kubo, S.; Matsuzaki, T.; Nishida, K.; Kurosaka, M.; Kuroda, R. The overexpression of SIRT1 inhibited osteoarthritic gene expression changes induced by interleukin-1β in human chondrocytes. *J. Orthop. Res.* **2013**, *31*, 531–537. [CrossRef] [PubMed]
41. Matsuzaki, T.; Matsushita, T.; Takayama, K.; Matsumoto, T.; Nishida, K.; Kuroda, R.; Kurosaka, M. Disruption of Sirt1 in chondrocytes causes accelerated progression of osteoarthritis under mechanical stress and during ageing in mice. *Ann. Rheum. Dis.* **2014**, *73*, 1397–1404. [CrossRef] [PubMed]
42. Petursson, F.; Husa, M.; June, R.; Lotz, M.; Terkeltaub, R.; Liu-Bryan, R. Linked decreases in liver kinase B1 and AMP-activated protein kinase activity modulate matrix catabolic responses to biomechanical injury in chondrocytes. *Arthritis Res. Ther.* **2013**, *15*, R77. [CrossRef] [PubMed]
43. Shakibaei, M.; Csaki, C.; Nebrich, S.; Mobasheri, A. Resveratrol suppresses interleukin-1β-induced inflammatory signaling and apoptosis in human articular chondrocytes: Potential for use as a novel nutraceutical for the treatment of osteoarthritis. *Biochem. Pharmacol.* **2008**, *76*, 1426–1439. [CrossRef] [PubMed]
44. Dave, M.; Attur, M.; Palmer, G.; Al-Mussawir, H.E.; Kennish, L.; Patel, J.; Abramson, S.B. The antioxidant resveratrol protects against chondrocyte apoptosis via effects on mitochondrial polarization and ATP production. *Arthritis Rheumatol.* **2008**, *58*, 2786–2797. [CrossRef] [PubMed]
45. Lei, M.; Wang, J.G.; Xiao, D.M.; Fan, M.; Wang, D.P.; Xiong, J.Y.; Chen, Y.; Ding, Y.; Liu, S.L. Resveratrol inhibits interleukin 1β-mediated inducible nitric oxide synthase expression in articular chondrocytes by activating SIRT1 and thereby suppressing nuclear factor-κB activity. *Eur. J. Pharmacol.* **2012**, *674*, 73–79. [CrossRef] [PubMed]

46. Wang, J.; Gao, J.S.; Chen, J.W.; Li, F.; Tian, J. Effect of resveratrol on cartilage protection and apoptosis inhibition in experimental osteoarthritis of rabbit. *Rheumatol. Int.* **2012**, *32*, 1541–1548. [CrossRef] [PubMed]
47. Rothwell, A.G.; Bentley, G. Chondrocyte multiplication in osteoarthritic articular cartilage. *J. Bone Jt. Surg.* **1973**, *55*, 588–594.
48. Carames, B.; Taniguchi, N.; Otsuki, S.; Blanco, F.J.; Lotz, M. Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. *Arthritis Rheumatol.* **2010**, *62*, 791–801. [CrossRef] [PubMed]
49. Dvir-Ginzberg, M.; Gagarina, V.; Lee, E.J.; Booth, R.; Gabay, O.; Hall, D.J. Tumor necrosis factor α-mediated cleavage and inactivation of SirT1 in human osteoarthritic chondrocytes. *Arthritis Rheumatol.* **2011**, *63*, 2363–2373. [CrossRef] [PubMed]
50. Dvir-Ginzberg, M.; Gagarina, V.; Lee, E.J.; Hall, D.J. Regulation of cartilage-specific gene expression in human chondrocytes by SirT1 and nicotinamide phosphoribosyltransferase. *J. Biol. Chem.* **2008**, *283*, 36300–36310. [CrossRef] [PubMed]
51. Zhao, X.; Petursson, F.; Viollet, B.; Lotz, M.; Terkeltaub, R.; Liu-Bryan, R. Peroxisome proliferator-activated receptor gamma coactivator 1α and FoxO3a mediate chondroprotection by AMP-activated protein kinase. *Arthritis Rheumatol.* **2014**, *66*, 3073–3082. [CrossRef] [PubMed]
52. Miyazaki, S.; Kakutani, K.; Yurube, T.; Maeno, K.; Takada, T.; Zhang, Z.; Kurakawa, T.; Terashima, Y.; Ito, M.; Ueha, T.; et al. Recombinant human SIRT1 protects against nutrient deprivation-induced mitochondrial apoptosis through autophagy induction in human intervertebral disc nucleus pulposus cells. *Arthritis Res. Ther.* **2015**, *17*, 253. [CrossRef] [PubMed]
53. Takayama, K.; Kawakami, Y.; Kobayashi, M.; Greco, N.; Cummins, J.H.; Matsushita, T.; Kuroda, R.; Kurosaka, M.; Fu, F.H.; Huard, J. Local intra-articular injection of rapamycin delays articular cartilage degeneration in a murine model of osteoarthritis. *Arthritis Res. Ther.* **2014**, *16*, 482. [CrossRef] [PubMed]
54. Caramés, B.; Kiosses, W.B.; Akasaki, Y.; Brinson, D.C.; Eap, W.; Koziol, J.; Lotz, M.K. Glucosamine activates autophagy in vitro and in vivo. *Arthritis Rheumatol.* **2013**, *65*, 1843–1852. [CrossRef] [PubMed]
55. Bruyere, O.; Altman, R.D.; Reginster, J.Y. Efficacy and safety of glucosamine sulfate in the management of osteoarthritis: Evidence from real-life setting trials and surveys. *Semin. Arthritis Rheum.* **2016**, *45*, S12–S17. [CrossRef] [PubMed]
56. Lee, Y.H.; Woo, J.H.; Choi, S.J.; Ji, J.D.; Song, G.G. Effect of glucosamine or chondroitin sulfate on the osteoarthritis progression: A meta-analysis. *Rheumatol. Int.* **2010**, *30*, 357–363. [CrossRef] [PubMed]
57. Sawitzke, A.D.; Shi, H.; Finco, M.F.; Dunlop, D.D.; Bingham, C.O., 3rd; Harris, C.L.; Singer, N.G.; Bradley, J.D.; Silver, D.; Jackson, C.G.; et al. The effect of glucosamine and/or chondroitin sulfate on the progression of knee osteoarthritis: A report from the glucosamine/chondroitin arthritis intervention trial. *Arthritis Rheumatol.* **2008**, *58*, 3183–3191. [CrossRef] [PubMed]
58. Herrero-Beaumont, G.; Ivorra, J.A.; Del Carmen Trabado, M.; Blanco, F.J.; Benito, P.; Martin-Mola, E.; Paulino, J.; Marenco, J.L.; Porto, A.; Laffon, A.; et al. Glucosamine sulfate in the treatment of knee osteoarthritis symptoms: A randomized, double-blind, placebo-controlled study using acetaminophen as a side comparator. *Arthritis Rheumatol.* **2007**, *56*, 555–567. [CrossRef] [PubMed]
59. Reginster, J.Y.; Deroisy, R.; Rovati, L.C.; Lee, R.L.; Lejeune, E.; Bruyere, O.; Giacovelli, G.; Henrotin, Y.; Dacre, J.E.; Gossett, C. Long-term effects of glucosamine sulphate on osteoarthritis progression: A randomised, placebo-controlled clinical trial. *Lancet* **2001**, *357*, 251–256. [CrossRef]
60. El-Arman, M.M.; El-Fayoumi, G.; El-Shal, E.; El-Boghdady, I.; El-Ghaweet, A. Aggrecan and cartilage oligomeric matrix protein in serum and synovial fluid of patients with knee osteoarthritis. *HSS J.* **2010**, *6*, 171–176. [CrossRef] [PubMed]
61. Pratta, M.A.; Su, J.L.; Leesnitzer, M.A.; Struglics, A.; Larsson, S.; Lohmander, L.S.; Kumar, S. Development and characterization of a highly specific and sensitive sandwich elisa for detection of aggrecanase-generated aggrecan fragments. *Osteoarthr. Cartil.* **2006**, *14*, 702–713. [CrossRef] [PubMed]
62. Struglics, A.; Larsson, S.; Pratta, M.A.; Kumar, S.; Lark, M.W.; Lohmander, L.S. Human osteoarthritis synovial fluid and joint cartilage contain both aggrecanase- and matrix metalloproteinase-generated aggrecan fragments. *Osteoarthr. Cartil.* **2006**, *14*, 101–113. [CrossRef] [PubMed]
63. Ratcliffe, A.; Beauvais, P.J.; Saed-Nejad, F. Differential levels of synovial fluid aggrecan aggregate components in experimental osteoarthritis and joint disuse. *J Orthop. Res.* **1994**, *12*, 464–473. [CrossRef] [PubMed]

64. Tiku, M.L.; Gupta, S.; Deshmukh, D.R. Aggrecan degradation in chondrocytes is mediated by reactive oxygen species and protected by antioxidants. *Free Radic. Res.* **1999**, *30*, 395–405. [CrossRef] [PubMed]
65. Leonardi, R.; Loreto, C.; Barbato, E.; Caltabiano, R.; Lombardo, C.; Musumeci, G.; Lo Muzio, L. MMP-13 (collagenase 3) localization in human temporomandibular joint discs with internal derangement. *Acta Histochem.* **2008**, *110*, 314–318. [CrossRef] [PubMed]
66. Lipari, L.; Gerbino, A. Expression of gelatinases (MMP-2, MMP-9) in human articular cartilage. *Int. J. Immunopathol. Pharmacol.* **2013**, *26*, 817–823. [CrossRef] [PubMed]
67. Wang, Y.; Tang, Z.; Xue, R.; Singh, G.K.; Shi, K.; Lv, Y.; Yang, L. Combined effects of TNF-α, IL-1β, and HIF-1α on MMP-2 production in ACL fibroblasts under mechanical stretch: An in vitro study. *J. Orthop. Res.* **2011**, *29*, 1008–1014. [CrossRef] [PubMed]
68. Leonardi, R.; Rusu, M.C.; Loreto, F.; Loreto, C.; Musumeci, G. Immunolocalization and expression of lubricin in the bilaminar zone of the human temporomandibular joint disc. *Acta Histochem.* **2012**, *114*, 1–5. [CrossRef] [PubMed]
69. Musumeci, G.; Castrogiovanni, P.; Trovato, F.M.; Imbesi, R.; Giunta, S.; Szychlinska, M.A.; Loreto, C.; Castorina, S.; Mobasheri, A. Physical activity ameliorates cartilage degeneration in a rat model of aging: A study on lubricin expression. *Scand. J. Med. Sci. Sports* **2015**, *25*, e222–e230. [CrossRef] [PubMed]
70. Musumeci, G.; Carnazza, M.L.; Loreto, C.; Leonardi, R.; Loreto, C. β-defensin-4 (HBD-4) is expressed in chondrocytes derived from normal and osteoarthritic cartilage encapsulated in PEGDA scaffold. *Acta Histochem.* **2012**, *114*, 805–812. [CrossRef] [PubMed]
71. Musumeci, G.; Carnazza, M.L.; Leonardi, R.; Loreto, C. Expression of β-defensin-4 in "an in vivo and ex vivo model" of human osteoarthritic knee meniscus. *Knee Surg. Sports Traumatol. Arthrosc.* **2012**, *20*, 216–222. [CrossRef] [PubMed]
72. Tagliafierro, L.; Officioso, A.; Sorbo, S.; Basile, A.; Manna, C. The protective role of olive oil hydroxytyrosol against oxidative alterations induced by mercury in human erythrocytes. *Food Chem. Toxicol.* **2015**, *82*, 59–63. [CrossRef] [PubMed]
73. Sun, W.; Wang, X.; Hou, C.; Yang, L.; Li, H.; Guo, J.; Huo, C.; Wang, M.; Miao, Y.; Liu, J.; et al. Oleuropein improves mitochondrial function to attenuate oxidative stress by activating the Nrf2 pathway in the hypothalamic paraventricular nucleus of spontaneously hypertensive rats. *Neuropharmacology* **2017**, *113*, 556–566. [CrossRef] [PubMed]
74. Maalej, A.; Mahmoudi, A.; Bouallagui, Z.; Fki, I.; Marrekchi, R.; Sayadi, S. Olive phenolic compounds attenuate deltamethrin-induced liver and kidney toxicity through regulating oxidative stress, inflammation and apoptosis. *Food Chem. Toxicol.* **2017**, *106*, 455–465. [CrossRef] [PubMed]
75. Kalaiselvan, I.; Dicson, S.M.; Kasi, P.D. Olive oil and its phenolic constituent tyrosol attenuates dioxin-induced toxicity in peripheral blood mononuclear cells via an antioxidant-dependent mechanism. *Nat. Prod. Res.* **2015**, *29*, 2129–2132. [CrossRef] [PubMed]
76. Cabrerizo, S.; De La Cruz, J.P.; Lopez-Villodres, J.A.; Munoz-Marin, J.; Guerrero, A.; Reyes, J.J.; Labajos, M.T.; Gonzalez-Correa, J.A. Role of the inhibition of oxidative stress and inflammatory mediators in the neuroprotective effects of hydroxytyrosol in rat brain slices subjected to hypoxia reoxygenation. *J. Nutr. Biochem.* **2013**, *24*, 2152–2157. [CrossRef] [PubMed]
77. Scoditti, E.; Nestola, A.; Massaro, M.; Calabriso, N.; Storelli, C.; De Caterina, R.; Carluccio, M.A. Hydroxytyrosol suppresses MMP-9 and COX-2 activity and expression in activated human monocytes via PKCα and PKCβ 1 inhibition. *Atherosclerosis* **2014**, *232*, 17–24. [CrossRef] [PubMed]
78. Pan, S.; Liu, L.; Pan, H.; Ma, Y.; Wang, D.; Kang, K.; Wang, J.; Sun, B.; Sun, X.; Jiang, H. Protective effects of hydroxytyrosol on liver ischemia/reperfusion injury in mice. *Mol. Nutr. Food Res.* **2013**, *57*, 1218–1227. [CrossRef] [PubMed]
79. Musumeci, G.; Maria Trovato, F.; Imbesi, R.; Castrogiovanni, P. Effects of dietary extra-virgin olive oil on oxidative stress resulting from exhaustive exercise in rat skeletal muscle: A morphological study. *Acta Histochem.* **2014**, *116*, 61–69. [CrossRef] [PubMed]
80. Takashima, T.; Sakata, Y.; Iwakiri, R.; Shiraishi, R.; Oda, Y.; Inoue, N.; Nakayama, A.; Toda, S.; Fujimoto, K. Feeding with olive oil attenuates inflammation in dextran sulfate sodium-induced colitis in rat. *J. Nutr. Biochem.* **2014**, *25*, 186–192. [CrossRef] [PubMed]

81. Silva, S.; Sepodes, B.; Rocha, J.; Direito, R.; Fernandes, A.; Brites, D.; Freitas, M.; Fernandes, E.; Bronze, M.R.; Figueira, M.E. Protective effects of hydroxytyrosol-supplemented refined olive oil in animal models of acute inflammation and rheumatoid arthritis. *J. Nutr. Biochem.* **2015**, *26*, 360–368. [CrossRef] [PubMed]
82. Sanchez-Fidalgo, S.; Villegas, I.; Aparicio-Soto, M.; Cardeno, A.; Rosillo, M.A.; Gonzalez-Benjumea, A.; Marset, A.; Lopez, O.; Maya, I.; Fernandez-Bolanos, J.G.; et al. Effects of dietary virgin olive oil polyphenols: Hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol on DSS-induced acute colitis in mice. *J. Nutr. Biochem.* **2015**, *26*, 513–520. [CrossRef] [PubMed]
83. Pirozzi, C.; Lama, A.; Simeoli, R.; Paciello, O.; Pagano, T.B.; Mollica, M.P.; Di Guida, F.; Russo, R.; Magliocca, S.; Canani, R.B.; et al. Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring duodenal integrity in a rat model of NAFLD. *J. Nutr. Biochem.* **2016**, *30*, 108–115. [CrossRef] [PubMed]
84. Lamy, S.; Ben Saad, A.; Zgheib, A.; Annabi, B. Olive oil compounds inhibit the paracrine regulation of TNF-α-induced endothelial cell migration through reduced glioblastoma cell cyclooxygenase-2 expression. *J. Nutr. Biochem.* **2016**, *27*, 136–145. [CrossRef] [PubMed]
85. Zheng, A.; Li, H.; Xu, J.; Cao, K.; Li, H.; Pu, W.; Yang, Z.; Peng, Y.; Long, J.; Liu, J.; et al. Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of db/db mice: Role of AMP-activated protein kinase activation. *Br. J. Nutr.* **2015**, *113*, 1667–1676. [CrossRef] [PubMed]
86. Kikusato, M.; Muroi, H.; Uwabe, Y.; Furukawa, K.; Toyomizu, M. Oleuropein induces mitochondrial biogenesis and decreases reactive oxygen species generation in cultured avian muscle cells, possibly via an up-regulation of peroxisome proliferator-activated receptor gamma coactivator-1α. *Anim. Sci. J.* **2016**, *87*, 1371–1378. [CrossRef] [PubMed]
87. Stiuso, P.; Bagarolo, M.L.; Ilisso, C.P.; Vanacore, D.; Martino, E.; Caraglia, M.; Porcelli, M.; Cacciapuoti, G. Protective effect of tyrosol and S-adenosylmethionine against ethanol-induced oxidative stress of Hepg2 cells involves sirtuin 1, p53 and Erk1/2 signaling. *Int. J. Mol. Sci.* **2016**, *17*, 622. [CrossRef] [PubMed]
88. Samuel, S.M.; Thirunavukkarasu, M. Akt/FOXO3a/SIRT1-mediated cardioprotection by n-tyrosol against ischemic stress in rat in vivo model of myocardial infarction: Switching gears toward survival and longevity. *J. Agric. Food Chem.* **2008**, *56*, 9692–9698. [CrossRef] [PubMed]
89. Lauretti, E.; Iuliano, L.; Praticò, D. Extra-virgin olive oil ameliorates cognition and neuropathology of the 3xTg mice: Role of autophagy. *Ann. Clin. Transl. Neurol.* **2017**, *4*, 564–574. [CrossRef] [PubMed]
90. Carnevale, R.; Pignatelli, P.; Nocella, C.; Loffredo, L.; Pastori, D.; Vicario, T.; Petruccioli, A.; Bartimoccia, S.; Violi, F. Extra virgin olive oil blunt post-prandial oxidative stress via NOX2 down-regulation. *Atherosclerosis* **2014**, *235*, 649–658. [CrossRef] [PubMed]
91. Camargo, A.; Rangel-Zuniga, O.A.; Haro, C.; Meza-Miranda, E.R.; Pena-Orihuela, P.; Meneses, M.E.; Marin, C.; Yubero-Serrano, E.M.; Perez-Martinez, P.; Delgado-Lista, J.; et al. Olive oil phenolic compounds decrease the postprandial inflammatory response by reducing postprandial plasma lipopolysaccharide levels. *Food Chem.* **2014**, *162*, 161–171. [CrossRef] [PubMed]
92. Bogani, P.; Galli, C.; Villa, M.; Visioli, F. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* **2007**, *190*, 181–186. [CrossRef] [PubMed]
93. Kim, H.A.; Cheon, E.J. Animal model of osteoarthritis. *J. Rheum. Dis.* **2012**, *19*, 239–247. [CrossRef]
94. Gong, D.Z.; Geng, C.Y.; Jiang, L.P.; Wang, L.H.; Yoshimura, H.; Zhong, L.F. Repair effect of olive leaf extract on experimental cartilaginous injuries in rabbits. *Chin. J. Pharmacol. Toxicol.* **2013**, *27*, 200–204.
95. Del Monaco, G.; Officioso, A.; D'Angelo, S.; La Cara, F.; Ionata, E.; Marcolongo, L.; Squillaci, G.; Maurelli, L.; Morana, A. Characterization of extra virgin olive oils produced with typical Italian varieties by their phenolic profile. *Food Chem.* **2015**, *184*, 220–228. [CrossRef] [PubMed]
96. Montano, A.; Hernandez, M.; Garrido, I.; Llerena, J.L.; Espinosa, F. Fatty acid and phenolic compound concentrations in eight different monovarietal virgin olive oils from Extremadura and the relationship with oxidative stability. *Int. J. Mol. Sci.* **2016**, *17*, E1960. [CrossRef] [PubMed]
97. Musumeci, G.; Trovato, F.M.; Pichler, K.; Weinberg, A.M.; Loreto, C.; Castrogiovanni, P. Extra-virgin olive oil diet and mild physical activity prevent cartilage degeneration in an osteoarthritis model: An in vivo and in vitro study on lubricin expression. *J. Nutr. Biochem.* **2013**, *24*, 2064–2075. [CrossRef] [PubMed]
98. Jay, G.D.; Waller, K.A. The biology of lubricin: Near frictionless joint motion. *Matrix Biol.* **2014**, *39*, 17–24. [CrossRef] [PubMed]

99. Mevel, E.; Merceron, C.; Vinatier, C.; Krisa, S.; Richard, T.; Masson, M.; Lesoeur, J.; Hivernaud, V.; Gauthier, O.; Abadie, J.; et al. Olive and grape seed extract prevents post-traumatic osteoarthritis damages and exhibits in vitro anti IL-1β activities before and after oral consumption. *Sci. Rep.* **2016**, *6*, 33527. [CrossRef] [PubMed]
100. Horcajada, M.N.; Sanchez, C.; Membrez Scalfo, F.; Drion, P.; Comblain, F.; Taralla, S.; Donneau, A.F.; Offord, E.A.; Henrotin, Y. Oleuropein or rutin consumption decreases the spontaneous development of osteoarthritis in the Hartley guinea pig. *Osteoarthr. Cartil.* **2015**, *23*, 94–102. [CrossRef] [PubMed]
101. Musumeci, G.; Mobasheri, A.; Trovato, F.M.; Szychlinska, M.A.; Imbesi, R.; Castrogiovanni, P. Post-operative rehabilitation and nutrition in osteoarthritis. *F1000Research* **2014**, *3*, 116. [CrossRef] [PubMed]
102. Bitler, C.M.; Matt, K.; Irving, M.; Hook, G.; Yusen, J.; Eagar, F.; Kirschner, K.; Walker, B.; Crea, R. Olive extract supplement decreases pain and improves daily activities in adults with osteoarthritis and decreases plasma homocysteine in those with rheumatoid arthritis. *Nutr. Res.* **2007**, *27*, 470–477. [CrossRef]
103. Bohlooli, S.; Jastan, M.; Nakhostin-Roohi, B.; Mohammadi, S.; Baghaei, Z. A pilot double-blinded, randomized, clinical trial of topical virgin olive oil versus piroxicam gel in osteoarthritis of the knee. *J. Clin. Rheumatol.* **2012**, *18*, 99–101. [CrossRef] [PubMed]
104. Takeda, R.; Koike, T.; Taniguchi, I.; Tanaka, K. Double-blind placebo-controlled trial of hydroxytyrosol of *Olea europaea* on pain in gonarthrosis. *Phytomedicine* **2013**, *20*, 861–864. [CrossRef] [PubMed]
105. Gelmini, F.; Ruscica, M.; MacChi, C.; Bianchi, V.; Maffei Facino, R.; Beretta, G.; Magni, P. Unsaponifiable fraction of unripe fruits of *Olea europaea*: An interesting source of anti-inflammatory constituents. *Planta Med.* **2016**, *82*, 273–278. [CrossRef] [PubMed]
106. Nsir, H.; Szychlinska, M.A.; Cardile, V.; Graziano, A.C.E.; Avola, R.; Esafi, H.; Bendini, A.; Zarouk, M.; Loreto, C.; Rapisarda, V.; et al. Polar and apolar extra virgin olive oil and leaf extracts as a promising anti-inflammatory natural treatment for osteoarthritis. *Acta Histochem.* **2017**, *119*, 407–416. [CrossRef] [PubMed]
107. Iacono, A.; Gomez, R.; Sperry, J.; Conde, J.; Bianco, G.; Meli, R.; Gomez-Reino, J.J.; Smith, A.B., III; Gualillo, O. Effect of oleocanthal and its derivatives on inflammatory response induced by lipopolysaccharide in a murine chondrocyte cell line. *Arthritis Rheumatol.* **2010**, *62*, 1675–1682. [CrossRef] [PubMed]
108. Scotece, M.; Gómez, R.; Conde, J.; Lopez, V.; Gómez-Reino, J.J.; Lago, F.; Smith, A.B., III; Gualillo, O. Further evidence for the anti-inflammatory activity of oleocanthal: Inhibition of MIP-1α and IL-6 in J774 macrophages and in ATDC5 chondrocytes. *Life Sci.* **2012**, *91*, 1229–1235. [CrossRef] [PubMed]
109. Facchini, A.; Cetrullo, S.; D'Adamo, S.; Guidotti, S.; Minguzzi, M.; Facchini, A.; Borzi, R.M.; Flamigni, F. Hydroxytyrosol prevents increase of osteoarthritis markers in human chondrocytes treated with hydrogen peroxide or growth-related oncogene α. *PLoS ONE* **2014**, *9*, e109724. [CrossRef] [PubMed]
110. D'Adamo, S.; Cetrullo, S.; Guidotti, S.; Borzi, R.M.; Flamigni, F. Hydroxytyrosol modulates the levels of microRNA-9 and its target sirtuin-1 thereby counteracting oxidative stress-induced chondrocyte death. *Osteoarthr. Cartil.* **2017**, *25*, 600–610. [CrossRef] [PubMed]
111. Carames, B.; López de Figueroa, P.; Ribeiro, M.; Calamia, V.; Romero, C.R.; Blanco, F.J. Deficient autophagy induces premature senescence in aging and osteoarthritis. *Osteoarthr. Cartil.* **2015**, *23*, A33–A34. [CrossRef]
112. Li, Y.S.; Zhang, F.J.; Zeng, C.; Luo, W.; Xiao, W.F.; Gao, S.G.; Lei, G.H. Autophagy in osteoarthritis. *Jt. Bone Spine* **2016**, *83*, 143–148. [CrossRef] [PubMed]
113. Cetrullo, S.; D'Adamo, S.; Guidotti, S.; Borzi, R.M.; Flamigni, F. Hydroxytyrosol prevents chondrocyte death under oxidative stress by inducing autophagy through sirtuin 1-dependent and -independent mechanisms. *Biochim. Biophys. Acta* **2016**, *1860*, 1181–1191. [CrossRef] [PubMed]
114. Komatsu, M.; Ichimura, Y. Physiological significance of selective degradation of p62 by autophagy. *FEBS Lett.* **2010**, *584*, 1374–1378. [CrossRef] [PubMed]
115. Rosillo, M.A.; Sanchez-Hidalgo, M.; Gonzalez-Benjumea, A.; Fernandez-Bolanos, J.G.; Lubberts, E.; Alarcon-de-la-Lastra, C. Preventive effects of dietary hydroxytyrosol acetate, an extra virgin olive oil polyphenol in murine collagen-induced arthritis. *Mol. Nutr. Food Res.* **2015**, *59*, 2537–2546. [CrossRef] [PubMed]
116. St-Laurent-Thibault, C.; Arseneault, M.; Longpre, F.; Ramassamy, C. Tyrosol and hydroxytyrosol, two main components of olive oil, protect N2a cells against amyloid-β-induced toxicity. Involvement of the NF-κB signaling. *Curr. Alzheimer Res.* **2011**, *8*, 543–551. [CrossRef] [PubMed]

117. Miro-Casas, E.; Covas, M.I.; Fito, M.; Farre-Albadalejo, M.; Marrugat, J.; de la Torre, R. Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. *Eur. J. Clin. Nutr.* **2003**, *57*, 186–190. [CrossRef] [PubMed]
118. Vissers, M.N.; Zock, P.L.; Roodenburg, A.J.; Leenen, R.; Katan, M.B. Olive oil phenols are absorbed in humans. *J. Nutr.* **2002**, *132*, 409–417. [PubMed]
119. Tuck, K.L.; Freeman, M.P.; Hayball, P.J.; Stretch, G.L.; Stupans, I. The in vivo fate of hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and oral dosing of labeled compounds to rats. *J. Nutr.* **2001**, *131*, 1993–1996. [PubMed]
120. Miro-Casas, E.; Covas, M.I.; Farre, M.; Fito, M.; Ortuno, J.; Weinbrenner, T.; Roset, P.; de la Torre, R. Hydroxytyrosol disposition in humans. *Clin. Chem.* **2003**, *49*, 945–952. [CrossRef] [PubMed]
121. Visioli, F.; Galli, C.; Bornet, F.; Mattei, A.; Patelli, R.; Galli, G.; Caruso, D. Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett.* **2000**, *468*, 159–160. [CrossRef]
122. Parkinson, L.; Cicerale, S. The health benefiting mechanisms of virgin olive oil phenolic compounds. *Molecules* **2016**, *21*, 1734. [CrossRef] [PubMed]
123. Nair, A.B.; Jacob, S. A simple practice guide for dose conversion between animals and human. *J. Basic Clin. Pharm.* **2016**, *7*, 27–31. [CrossRef] [PubMed]
124. Ng, S.F.; Tan, L.S.; Buang, F. Transdermal anti-inflammatory activity of bilayer film containing olive compound hydroxytyrosol: Physical assessment, in vivo dermal safety and efficacy study in Freund's adjuvant-induced arthritic rat model. *Drug Dev. Ind. Pharm.* **2017**, *43*, 108–119. [CrossRef] [PubMed]
125. Siddique, M.I.; Katas, H.; Amin, M.; Ng, S.F.; Zulfakar, M.H.; Buang, F.; Jamil, A. Minimization of local and systemic adverse effects of topical glucocorticoids by nanoencapsulation: In vivo safety of hydrocortisone-hydroxytyrosol loaded chitosan nanoparticles. *J. Pharm. Sci.* **2015**, *104*, 4276–4286. [CrossRef] [PubMed]
126. Flaiz, L.; Freire, M.; Cofrades, S.; Mateos, R.; Weiss, J.; Jimenez-Colmenero, F.; Bou, R. Comparison of simple, double and gelled double emulsions as hydroxytyrosol and *n*-3 fatty acid delivery systems. *Food Chem.* **2016**, *213*, 49–57. [CrossRef] [PubMed]
127. Cofrades, S.; Bou, R. Bioaccessibility of hydroxytyrosol and *n*-3 fatty acids as affected by the delivery system: Simple, double and gelled double emulsions. *J. Food Sci. Technol.* **2017**, *54*, 1785–1793. [CrossRef] [PubMed]
128. Junker, S.; Frommer, K.W.; Krumbholz, G.; Tsiklauri, L.; Gerstberger, R.; Rehart, S.; Steinmeyer, J.; Rickert, M.; Wenisch, S.; Schett, G.; et al. Expression of adipokines in osteoarthritis osteophytes and their effect on osteoblasts. *Matrix Biol.* **2017**, *62*, 75–91. [CrossRef] [PubMed]
129. Nakajima, R.; Inada, H.; Koike, T.; Yamano, T. Effects of leptin to cultured growth plate chondrocytes. *Horm. Res. Paediatr.* **2003**, *60*, 91–98. [CrossRef]
130. Presle, N.; Pottie, P.; Dumond, H.; Guillaume, C.; Lapicque, F.; Pallu, S.; Mainard, D.; Netter, P.; Terlain, B. Differential distribution of adipokines between serum and synovial fluid in patients with osteoarthritis. Contribution of joint tissues to their articular production. *Osteoarthr. Cartil.* **2006**, *14*, 690–695. [CrossRef] [PubMed]
131. Reseland, J.E.; Syversen, U.; Bakke, I.; Qvigstad, G.; Eide, L.G.; Hjertner, Ø.; Gordeladze, J.O.; Drevon, C.A. Leptin is expressed in and secreted from primary cultures of human osteoblasts and promotes bone mineralization. *J. Bone Miner. Res.* **2001**, *16*, 1426–1433. [CrossRef] [PubMed]
132. Dumond, H.; Presle, N.; Terlain, B.; Mainard, D.; Loeuille, D.; Netter, P.; Pottie, P. Evidence for a key role of leptin in osteoarthritis. *Arthritis Rheumatol.* **2003**, *48*, 3118–3129. [CrossRef] [PubMed]
133. Vuolteenaho, K.; Koskinen, A.; Kukkonen, M.; Nieminen, R.; Paivarinta, U.; Moilanen, T.; Moilanen, E. Leptin enhances synthesis of proinflammatory mediators in human osteoarthritic cartilage—Mediator role of NO in leptin-induced PGE_2, IL-6, and IL-8 production. *Mediat. Inflamm.* **2009**, *2009*, 345838. [CrossRef] [PubMed]
134. Simopoulou, T.; Malizos, K.N.; Iliopoulos, D.; Stefanou, N.; Papatheodorou, L.; Ioannou, M.; Tsezou, A. Differential expression of leptin and leptin's receptor isoform (Ob-Rb) mRNA between advanced and minimally affected osteoarthritic cartilage; effect on cartilage metabolism. *Osteoarthr. Cartil.* **2007**, *15*, 872–883. [CrossRef] [PubMed]

135. Cnop, M.; Havel, P.J.; Utzschneider, K.M.; Carr, D.B.; Sinha, M.K.; Boyko, E.J.; Retzlaff, B.M.; Knopp, R.H.; Brunzell, J.D.; Kahn, S.E. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: Evidence for independent roles of age and sex. *Diabetologia* **2003**, *46*, 459–469. [CrossRef] [PubMed]
136. Chen, T.H.; Chen, L.; Hsieh, M.S.; Chang, C.P.; Chou, D.T.; Tsai, S.H. Evidence for a protective role for adiponectin in osteoarthritis. *Biochim. Biophys. Acta* **2006**, *1762*, 711–718. [CrossRef] [PubMed]
137. Koskinen, A.; Juslin, S.; Nieminen, R.; Moilanen, T.; Vuolteenaho, K.; Moilanen, E. Adiponectin associates with markers of cartilage degradation in osteoarthritis and induces production of proinflammatory and catabolic factors through mitogen-activated protein kinase pathways. *Arthritis Res. Ther.* **2011**, *13*, R184. [CrossRef] [PubMed]
138. Choi, H.M.; Lee, Y.A.; Lee, S.H.; Hong, S.J.; Hahm, D.H.; Choi, S.Y.; Yang, H.I.; Yoo, M.C.; Kim, K.S. Adiponectin may contribute to synovitis and joint destruction in rheumatoid arthritis by stimulating vascular endothelial growth factor, matrix metalloproteinase-1, and matrix metalloproteinase-13 expression in fibroblast-like synoviocytes more than proinflammatory mediators. *Arthritis Res. Ther.* **2009**, *11*, R161. [PubMed]
139. Lago, R.; Gomez, R.; Otero, M.; Lago, F.; Gallego, R.; Dieguez, C.; Gomez-Reino, J.J.; Gualillo, O. A new player in cartilage homeostasis: Adiponectin induces nitric oxide synthase type II and pro-inflammatory cytokines in chondrocytes. *Osteoarthr. Cartil.* **2008**, *16*, 1101–1109. [CrossRef] [PubMed]
140. Scoditti, E.; Massaro, M.; Carluccio, M.A.; Pellegrino, M.; Wabitsch, M.; Calabriso, N.; Storelli, C.; De Caterina, R. Additive regulation of adiponectin expression by the mediterranean diet olive oil components oleic acid and hydroxytyrosol in human adipocytes. *PLoS ONE* **2015**, *10*, e0128218. [CrossRef] [PubMed]
141. Kabiri, A.; Hosseinzadeh-Attar, M.J.; Haghighatdoost, F.; Eshraghian, M.; Esmaillzadeh, A. Impact of olive oil-rich diet on serum omentin and adiponectin levels: A randomized cross-over clinical trial among overweight women. *Int. J. Food Sci. Nutr.* **2017**, *68*, 560–568. [CrossRef] [PubMed]
142. Tantavisut, S.; Tanavalee, A.; Honsawek, S.; Suantawee, T.; Ngarmukos, S.; Adisakwatana, S.; Callaghan, J.J. Effect of vitamin E on oxidative stress level in blood, synovial fluid, and synovial tissue in severe knee osteoarthritis: A randomized controlled study. *BMC Musculoskelet. Disord.* **2017**, *18*, 281. [CrossRef] [PubMed]
143. Bastiaansen-Jenniskens, Y.M.; Siawash, M.; van de Lest, C.H.; Verhaar, J.A.; Kloppenburg, M.; Zuurmond, A.M.; Stojanovic-Susulic, V.; Van Osch, G.J.; Clockaerts, S. Monounsaturated and saturated, but not *n*-6 polyunsaturated fatty acids decrease cartilage destruction under inflammatory conditions: A preliminary study. *Cartilage* **2013**, *4*, 321–328. [CrossRef] [PubMed]
144. Li, G.; Yin, J.; Gao, J.; Cheng, T.S.; Pavlos, N.J.; Zhang, C.; Zheng, M.H. Subchondral bone in osteoarthritis: Insight into risk factors and microstructural changes. *Arthritis Res. Ther.* **2013**, *15*, 223. [CrossRef] [PubMed]
145. Chin, K.-Y.; Ima-Nirwana, S. Olives and bone: A green osteoporosis prevention option. *Int. J. Environ. Res. Public Health* **2016**, *13*, 755. [CrossRef] [PubMed]
146. Bellido, M.; Lugo, L.; Roman-Blas, J.A.; Castaneda, S.; Calvo, E.; Largo, R.; Herrero-Beaumont, G. Improving subchondral bone integrity reduces progression of cartilage damage in experimental osteoarthritis preceded by osteoporosis. *Osteoarthr. Cartil.* **2011**, *19*, 1228–1236. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

nutrients

MDPI

Article

Association between Dietary Phenolic Acids and Hypertension in a Mediterranean Cohort

Justyna Godos *, Dario Sinatra, Isabella Blanco, Serena Mulè, Melania La Verde and Marina Marranzano

Department of Medical and Surgical Sciences and Advanced Technologies "G.F. Ingrassia", University of Catania, 95123 Catania, Italy; sinatradario@gmail.com (D.S.); dott.ssa.blanco.isabella@gmail.com (I.B.); serenamule86@gmail.com (S.M.); melanialaverde@yahoo.it (M.L.V.); marranz@unict.it (M.M.)

* Correspondence: justyna.godos@student.uj.edu.pl; Tel.: +39-095-378-2180

Received: 15 September 2017; Accepted: 22 September 2017; Published: 27 September 2017

Abstract: Background: Certain foods rich in phenolic acids have been shown to reduce the risk of hypertension, but evidence from epidemiological studies focused on dietary phenolic acid intake is scarce. The aim of this study was to determine the association between dietary phenolic acid intake, as well as their major food sources, and hypertension in a Mediterranean cohort. Methods: Demographic and dietary data of 2044 adults living in Southern Italy were collected. Food frequency questionnaires and Phenol-Explorer were used to calculate dietary intake of polyphenols. Multivariate logistic regression analyses were used to test associations. Results: The mean intake of total phenolic acids in the cohort was 362.6 mg/day. Individuals in the highest quartile of phenolic acid intake (median intake = 522.2 mg/day) were less likely to have hypertension (OR (odds ratio) = 0.68, 95% CI (confidence interval): 0.46, 1.00). When taking into account individual subclasses of phenolic acids, only hydroxyphenylacetic acid was inversely associated with hypertension (highest vs. lowest quartile, OR = 0.63, 95% CI: 0.40, 0.96). Among dietary sources of phenolic acids considered in the analysis, only beer was significantly inversely associated with hypertension (highest vs. lowest quartile, OR = 0.32, 95% CI: 0.15, 0.68). Conclusions: The findings of this study suggest that dietary phenolic acids may be inversely associated with hypertension, irrespectively of their dietary source.

Keywords: phenolic acids; polyphenols; fruit; vegetable; nuts; hypertension; cohort study; Mediterranean area

1. Introduction

Plant-based dietary patterns have been associated with better metabolic profile and lower risk of non-communicable diseases [1–3]. Key components, such as fruits and vegetables, but also tea, have been associated with beneficial effects on health [4]. The potential effects of other foods, such as beer, wine, nuts and coffee, have been historically controversial due to their content of compounds (such as ethanol, fats, and caffeine, respectively) that are known to exert detrimental effects on health. However, their impact on health has been recently reconsidered [5–7]: indeed, summary results of epidemiological studies show an overall decreased mortality risk associated with moderate consumption of such foods and beverages [8–10]. The biological rationale of this potential benefit may rely on a better knowledge of the role of antioxidants in the diet and potential risk modification depending on the quantity and quality of compounds representative of the aforementioned foods and beverages [11,12].

Dietary polyphenols have been recently studied as potential mediators of the beneficial effects of antioxidant-rich foods on certain health outcomes. With regard to anti-hypertensive effects, dietary polyphenols have demonstrated antioxidant, anti-inflammatory, and antithrombotic properties, as well as their capacity to affect NO (nitric oxide) bioavailability [13]. Among common classes of polyphenols in tea, nuts, coffee, olive oil, and alcoholic beverages, phenolic acids are compounds containing a

phenolic ring and an organic carboxylic acid function (C6-C1 skeleton) [14]. Observational studies on the association between phenolic acids and cardio-metabolic risk factors are scarce. Results from cross-sectional studies are not univocal, as some studies showed no significant findings [15] while others showed a relation between phenolic acid intake and blood pressure as component of metabolic syndrome [16,17]. Only one prospective study including phenolic acid exposure, and specifically assessing hypertension as an outcome, has been published to date, showing a significant lower risk of disease [18]. Overall, evidence on such matters is still limited and more investigations are needed to confirm the consistency of results and better identify potential dietary sources of phenolic acids that may play a role in decreasing the risk of hypertension. Therefore, the aim of this study was to assess the association between dietary phenolic acid consumption, as well as their major food sources, and hypertension in a cohort of adults living in the Mediterranean area.

2. Materials and Methods

2.1. Study Design and Population

The MEAL (Mediterranean healthy Eating, Ageing, and Lifestyle) study is an observational study primarily designed to investigate the relationship between nutritional habits characterizing the classical Mediterranean lifestyle and non-communicable diseases. The baseline data included a random sample of the general population including 2044 men and women aged 18 years or older, stratified by gender and 10-year age groups, randomly selected in the main districts of the city of Catania, Southern Italy. The enrollment and data collection was performed between 2014 and 2015 through the selection among the lists of registered patients of a pool of general practitioners. Full details of the study protocol are published elsewhere [19]. All participants were informed about the aims of the study and provided written informed consent. All of the study procedures were carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association. The study protocol has been approved by the concerning ethical committee.

2.2. Data Collection

An electronic data collection was performed by a face-to-face computer-assisted personal interview using tablet computers. In order to visualize the response options, participants were provided with a paper copy of the questionnaire. However, final answers were registered by the interviewer directly on the digital device (tablet computer). The demographic data comprised gender, age at recruitment, latest educational degree achieved, occupation (specifies the character of the most important employment during the year before the investigation) or last occupation before retirement, and marital status; educational status was categorized as (i) low (primary/secondary); (ii) medium (high school); and (iii) high (university). Occupational status was categorized as (i) unemployed; (ii) low (unskilled workers); (iii) medium (partially-skilled workers); and (iv) high (skilled workers) [20]. Physical activity status was evaluated through the International Physical Activity Questionnaires (IPAQ) [21], which included a set of questionnaires (5 domains) investigating the time spent being physically active in the last seven days: based on the IPAQ guidelines, final scores allow the physical activity level to be categorized as (i) low; (ii) moderate; and (iii) high. Smoking status was categorized as (i) non-smoker; (ii) ex-smoker; and (iii) current smoker. Alcohol consumption was categorized as (i) none; (ii) moderate drinker (0.1–12 g/day); and (iii) regular drinker (>12 g/day).

2.3. Dietary Assessment

The dietary assessment was performed by the administration of two food frequency questionnaires (a long and a short version) that were previously tested for validity and reliability for the Sicilian population [22,23]. Subsequently, the identification of the food intake, the energy content, as well as the macro- and micro-nutrient intake were obtained through comparison with food composition tables of the Research Center for Foods and Nutrition [24]. Intake of seasonal foods

referred to consumption during the period in which the food was available and then adjusted by its proportional intake in one year. FFQs (Food Frequency Questionnaires) with unreliable intakes (<1000 or >6000 kcal/day) were excluded from the analyses (n = 107) leaving a total of 1937 individuals included in the analysis.

2.4. Estimation of Polyphenol Intake

The process of the estimation of polyphenol intake was described in detail elsewhere [25]. Briefly, data on the polyphenol content in foods were obtained from the Phenol-Explorer database (www.phenol-explorer.eu) [26]. A new version of the Phenol-Explorer database containing data on the effects of cooking and food processing on polyphenol contents was used whenever possible in order to apply polyphenol-specific retention factors [27]. Foods that contained no polyphenols were excluded from the calculation, leaving a total of 75 items included in the analyses. Weight loss or gain during cooking was corrected using yield factors [28]. The average food consumption was calculated (in g or mL) by following the standard portion sizes used in the study and then converted in 24-h intake. Finally, a search was carried out in the Phenol-Explorer database to retrieve mean content values for all polyphenols contained in the selected foods. Next, polyphenol intake from each food was calculated by multiplying the content of each polyphenol by the daily consumption of each food. The main classes of polyphenols (flavonoids, phenolic acids, lignans, stilbenes, others) and the total polyphenol intake was estimated by the sum of the previous; additional subclass and selected individual polyphenols were also estimated. Finally, total and individual classes of polyphenol intake were adjusted for total energy intake (kcal/day) using the residual method [29].

2.5. Anthropometric Measurements and Outcome Ascertainment

Height was measured to the nearest 0.5 cm without shoes, with the back square against the wall tape, eyes looking straight ahead, with a right-angle triangle resting on the scalp and against the wall. Waist circumference (centimeters) was measured midway between the 12th rib and the iliac crest. Body mass index (BMI) was calculated, and patients were categorized as under/normal weight (BMI < 25 kg/m^2), excess body weight (BMI $\geq$ 25 kg/m^2), and obese (BMI > 30 kg/m^2).

Arterial blood pressure was measured in sitting position and at least 5 min at rest at the end of the physical examination. Because of the possibility of differences in blood pressure measurement, the measurements were taken three times at the right arm relaxed and well supported by a table, with an angle of 45° from the trunk. A mean of the last two measurements was calculated and considered for inclusion in the database. Information from measurements was integrated with general practitioners' computerized records, as patients are diagnosed with disease by a specialist in order to obtain drug reimbursement. Patients were considered hypertensive when average systolic/diastolic blood pressure levels were higher or equal to 140/90 mm Hg, taking anti-hypertensive medications, or being previously diagnosed with hypertension.

2.6. Statistical Analysis

Frequencies are presented as absolute numbers and percentages; continuous variables are presented as means and standard deviations. Individuals were divided into quartiles of phenolic acid intake and the distribution of background characteristics were compared between groups. Differences were tested with the Chi-square test for categorical variables, ANOVA for continuous variables distributed normally, and the Kruskall-Wallis test for variables not distributed normally. Age- and energy-adjusted multivariate logistic regression models were used to test the association between variables of exposure (including total phenolic acids and individual subclasses and specific compound intake) and having hypertension; additional multivariate models adjusted for all other background characteristics (age, sex, BMI, educational and occupational status, smoking and alcohol drinking habits, physical activity level, and dietary sodium, potassium, calcium, and magnesium intake) were also performed to test whether the association retrieved was independent from the aforementioned

potential confounding factors. Finally, an additional analysis on the association between major food sources of phenolic acids and hypertension was finally performed based on the results of a previous study in which they were identified as coffee, nuts, tea, olive oil, red wine, white wine, and beer [25]. All reported p-values were based on two-sided tests and compared to a significance level of 5%. SPSS 17 (SPSS Inc., Chicago, IL, USA) software was used for all the statistical calculations.

3. Results

Baseline characteristics of 1936 participants included in the analysis according to quartiles of phenolic acid intake are shown in Table 1. Among individuals in the highest category of phenolic acid intake there was a higher percentage of regular and moderate drinkers, current and ex-smokers, and individuals with lower educational and occupational level (higher percentage of unemployed) (Table 1). No further significant differences were found regarding sex, age, and physical activity (Table 1). With increasing intake of phenolic acids, a higher intake of micronutrients that might affect blood pressure (including sodium, potassium, calcium, and magnesium) was observed; in contrast, adherence to the Mediterranean diet did not follow a linear association with intake of phenolic acids, as a higher percentage of individuals' high adherence to this dietary pattern had intermediary consumption of phenolic acids (Table 1). All other major macro- and micronutrients were associated in a direct manner with intake of phenolic acids, suggesting a wide distribution in foods commonly consumed by participants not related to diet quality features (as identified with the Mediterranean diet) (Supplementary Table S1).

Table 1. Background characteristics by quartiles of dietary phenolic acids in the MEAL study sample (n = 1936); Q = quartile.

	Phenolic Acid Intake				
	Q1 (Median = 120.36)	**Q2 (Median = 205.39)**	**Q3 (Median = 307.70)**	**Q4 (Median = 522.26)**	p
Sex, n (%)					0.919
Male	186 (41.1)	197 (40.5)	215 (42.3)	206 (42.2)	
Female	267 (58.9)	290 (59.5)	293 (57.7)	282 (57.8)	
Educational level, n (%)					<0.001
Low	150 (33.1)	161 (33.1)	198 (39.0)	188 (38.5)	
Medium	141 (31.1)	218 (44.8)	177 (34.8)	184 (37.7)	
High	162 (35.8)	108 (22.2)	133 (26.2)	116 (23.8)	
Occupational level, n (%)					0.046
Unemployed	94 (22.8)	110 (27.0)	134 (29.7)	123 (31.9)	
Low	69 (16.7)	75 (18.4)	67 (14.9)	55 (14.2)	
Medium	111 (26.9)	95 (23.3)	131 (29.0)	103 (26.7)	
High	139 (33.7)	128 (31.4)	119 (26.4)	105 (27.2)	
Smoking status, n (%)					0.034
Non smoker	298 (65.8)	289 (59.3)	316 (62.2)	292 (59.8)	
Ex-smoker	85 (18.8)	131 (26.9)	132 (26.0)	117 (24.0)	
Current smoker	70 (15.5)	67 (13.8)	60 (11.8)	79 (16.2)	
Physical activity, n (%)					0.585
Low	85 (21.5)	87 (19.1)	75 (16.4)	82 (19.5)	
Medium	190 (48.0)	218 (47.9)	234 (51.3)	214 (50.8)	
High	121 (30.6)	150 (33.0)	147 (32.2)	125 (29.7)	
Alcohol intake, n (%)					<0.001
No	110 (24.2)	108 (22.2)	97 (19.1)	60 (12.3)	
Moderate	324 (71.4)	319 (65.5)	290 (57.1)	273 (55.9)	
Regular	20 (4.4)	60 (12.3)	121 (23.8)	155 (31.8)	
Age (years), mean (SD)	47.4 (19.3)	48.7 (18.2)	48.0 (16.0)	49.5 (16.9)	0.296
Sodium (mg/day), mean (SD)	2669 (864.2)	2868 (1074.2)	2793.2 (1174.3)	3093.8 (1224.1)	<0.001
Potassium(mg/day), mean (SD)	2892 (848.8)	3471 (1027.1)	3892.1(1306.1)	4392.4 (1713.6)	<0.001
Magnesium (mg/day), mean (SD)	311.1 (87.9)	382.5 (109.5)	416.2 (128.6)	467.5 (172.7)	<0.001
Calcium (mg/day), mean (SD)	690.5 (258.3)	755.6 (264.7)	808.3 (331.0)	951.6 (400.5)	<0.001
High adherence to the Mediterranean diet (high)	40 (14.5)	102 (37.1)	82 (29.8)	51 (18.5)	<0.001

The mean intake of phenolic acids in the cohort was 362.6 mg/day; when considering the most adjusted model (model 3), individuals in the highest quartile of total phenolic acid intake (median intake = 522.2 mg/day) were less likely to have hypertension (OR (odds ratio) = 0.65, 95% CI (confidence interval): 0.43, 0.98) despite there was no clear trend across quartiles of exposure (Table 2). The analysis of individual subclasses of phenolic acids and specific compounds revealed a significant association with hypertension only of hydroxycinnamic acid (highest vs. lowest category, OR = 0.52, 95% CI: 0.32, 0.95) and hydroxyphenylacetic acids (highest vs. lowest category, OR = 0.61, 95% CI: 0.39, 0.97). When analyses were stratified by sex, a significant inverse association between hydroxycinnamic and hydroxyphenylacetic acids and hypertension was found in women, but not in men, while an inverse association with caffeic acid was found in men (Supplementary Table S2).

Table 2. Association between total, main classes, and individual phenolic acids and hypertension.

	Phenolic Acid Intake				
	Q1	**Q2**	**Q3**	**Q4**	***p* for Trend**
Phenolic acids, mean (range), mg/day	112.12 (19.52, 156.19)	204.05 (156.28, 248.85)	311.00 (248.93, 385.09)	807.81 (386.07, 8361.62)	
No. of cases	224	278	257	217	
Model 1, OR (95% CI) [a]	1	1.27 (0.95, 1.70)	0.97 (0.73, 1.29)	0.72 (0.52, 0.98)	
Model 2, OR (95% CI) [b]	1	1.16 (0.80, 1.67)	0.86 (0.59, 1.24)	0.74 (0.48, 1.13)	
Model 3, OR (95% CI) [c]	1	1.08 (0.73, 1.60)	0.74 (0.49, 1.10)	0.65 (0.43, 0.98)	0.056
Hydroxybenzoic acids, mean (range), mg/day	12.81 (0.00, 47.36)	64.18 (47.73, 81.36)	136.38 (81.50, 258.37)	617.46 (258.59, 8265.42)	
No. of cases	231	282	241	222	
Model 1, OR (95% CI) [a]	1	1.49 (1.10, 2.00)	1.13 (0.84, 1.52)	0.85 (0.63, 1.15)	
Model 2, OR (95% CI) [b]	1	1.25 (0.89, 1.76)	0.78 (0.53, 1.13)	0.93 (0.64, 1.35)	
Model 3, OR (95% CI) [c]	1	1.21 (0.84, 1.75)	0.79 (0.52, 1.19)	0.93 (0.62, 1.40)	0.237
Hydroxycinammic acid, mean (range), mg/day	62.18 (14.72, 84.14)	106.72 (84.14, 128.85)	156.65 (128.87, 191.00)	271.05 (191.06, 836.62)	
No. of cases	223	261	267	225	
Model 1, OR (95% CI) [a]	1	1.17 (0.87, 1.57)	1.01 (0.75, 1.37)	0.85 (0.62, 1.17)	
Model 2, OR (95% CI) [b]	1	0.96 (0.66, 1.39)	0.74 (0.50, 1.09)	0.71 (0.46, 1.11)	
Model 3, OR (95% CI) [c]	1	0.82 (0.55, 1.21)	0.61 (0.40, 0.94)	0.52 (0.32, 0.85)	<0.001
Hydroxyphenylacetic acid, mean (range), mg/day	0.03 (0.00, 0.09)	0.15 (0.09, 0.23)	0.36 (0.23, 0.48)	1.31 (0.48, 13.52)	
No. of cases	226	264	248	238	
Model 1, OR (95% CI) [a]	1	1.34 (1.00, 1.81)	1.18 (0.88, 1.60)	1.00 (0.73, 1.36)	
Model 2, OR (95% CI) [b]	1	1.15 (0.80, 1.65)	0.98 (0.68, 1.42)	0.70 (0.46, 1.08)	
Model 3, OR (95% CI) [c]	1	0.95 (0.65, 1.39)	0.85 (0.57, 1.27)	0.61 (0.39, 0.97)	<0.001
Caffeic acid, mean (range), mg/day	0.41 (0.00, 0.60)	0.79 (0.60, 1.00)	1.43 (1.00, 2.10)	4.01 (2.11, 10.33)	
No. of cases	243	229	250	254	

Table 2. *Cont.*

	Phenolic Acid Intake				
	Q1	Q2	Q3	Q4	*p* for Trend
Model 1, OR (95% CI) [a]	1	0.71 (0.52, 0.95)	0.82 (0.61, 1.10)	0.88 (0.64, 1.22)	
Model 2, OR (95% CI) [b]	1	0.86 (0.59, 1.26)	0.92 (0.62, 1.37)	0.70 (0.40, 1.22)	
Model 3, OR (95% CI) [c]	1	0.99 (0.67, 1.50)	0.94 (0.61, 1.46)	0.83 (0.46, 1.50)	0.041
Cinnamic acid, mean (range), mg/day	0.02 (0.00, 0.08)	0.12 (0.08, 0.19)	0.28 (0.20, 0.43)	1.17 (0.43, 13.62)	
No. of cases	246	239	220	271	
Model 1, OR (95% CI) [a]	1	0.83 (0.61, 1.12)	0.88 (0.65, 1.20)	0.90 (0.67, 1.22)	
Model 2, OR (95% CI) [b]	1	0.70 (0.49, 0.99)	0.58 (0.40, 0.83)	0.81 (0.56, 1.16)	
Model 3, OR (95% CI) [c]	1	0.68 (0.47, 0.98)	0.56 (0.38, 0.84)	0.74 (0.50, 1.10)	0.631
Vanillic acid, mean (range), mg/day	0.05 (0.00, 0.08)	0.14 (0.08, 0.22)	0.35 (0.22, 0.54)	0.99 (0.54, 5.02)	
No. of cases	239	263	238	236	
Model 1, OR (95% CI) [a]	1	1.33 (0.98, 1.79)	0.95 (0.70, 1.28)	0.93 (0.68, 1.27)	
Model 2, OR (95% CI) [b]	1	1.26 (0.87, 1.83)	0.88 (0.60, 1.30)	0.75 (0.47, 1.18)	
Model 3, OR (95% CI) [c]	1	1.11 (0.75, 1.64)	0.91 (0.60, 1.39)	0.75 (0.46, 1.23)	0.427
Ferulic acid, mean (range), mg/day	0.55 (0.00, 0.93)	1.38 (0.93, 1.85)	2.69 (1.85, 4.04)	6.96 (4.05, 20.77)	
No. of cases	244	248	279	205	
Model 1, OR (95% CI) [a]	1	0.93 (0.69, 1.25)	1.19 (0.88, 1.60)	0.69 (0.50, 0.95)	
Model 2, OR (95% CI) [b]	1	1.14 (0.79, 1.64)	1.44 (1.01, 2.07)	0.78 (0.51, 1.18)	
Model 3, OR (95% CI) [c]	1	1.25 (0.84, 1.86)	1.76 (1.18, 2.61)	0.82 (0.52, 1.30)	0.398

[a] Model 1 adjusted for age (years, continuous), energy intake (kcal/day, continuous); [b] Model 2 = Model 1 + body mass index, smoking status (smokers, ex-smokers, non-smokers), alcohol consumption (0 g/day, <12 g/day, ≥12 g/day), physical activity level (low, medium, high), educational level (low, medium, high), occupational level (unemployed, low, medium, high), menopausal status (in women), fiber, sodium, potassium, magnesium, and calcium intake; [c] Model 3 = Model 2 + adherence to the Mediterranean diet. OR (odds ratio); CI (confidence interval).

Among major dietary sources of phenolic acids considered in this study, only beer was significantly inversely associated with hypertension (highest vs. lowest category, OR = 0.32, 95% CI: 0.15, 0.68; Table 3).

Table 3. Association between major food sources of phenolic acids and hypertension.

	Food group intake, OR (95% CI) [a]			
	Q1	**Q2**	**Q3**	**Q4**
Coffee [b]	1	0.85 (0.51, 1.44)	1.03 (0.66, 1.62)	0.70 (0.45, 1.08)
Nuts [c]	1	1.19 (0.90, 1.59)	1.07 (0.69, 1.67)	1.24 (0.73, 2.12)
Tea [d]	1	1.12 (0.86, 1.47)	0.75 (0.48, 1.17)	0.45 (0.17, 1.14)
Olive oil [e]	1	0.53 (0.22, 1.23)	0.50 (0.22, 1.10)	0.63 (0.29, 1.37)
Red wine [f]	1	0.75 (0.53, 1.06)	1.13 (0.52, 2.45)	0.74 (0.24, 2.25)
White wine [f]	1	0.72 (0.54, 0.95)	1.25 (0.38, 4.11)	-
Beer [g]	1	0.83 (0.58, 1.20)	0.51 (0.32, 0.81)	0.32 (0.15, 0.68)

[a] OR adjusted for sex, age (years, continuous), energy intake (kcal/day, continuous), smoking status (smokers, ex-smokers, non-smokers), alcohol consumption (0 g/day, <12 g/day, ≥12 g/day), physical activity level (low, medium, high), educational level (low, medium, high), occupational level (unemployed, low, medium, high), menopausal status (in women), fiber, sodium and potassium intake; [b] categories of coffee intake were as follow: Q1, none; Q2, <25 mL/day; Q3, 25–50 mL/day; Q4, >50 mL/day; [c] categories of nuts intake were as follow: Q1, none; Q2, <28 g/day; Q3, 28–56 g/day; Q4, >56 g/day; [d] categories of tea intake were as follow: Q1, none; Q2, <250 mL/day; Q3, 250–500 mL/day; Q4, >500 mL/day; [e] categories of olive oil intake were as follow: Q1, <2.5 mL/day; Q2, 2.5–4 mL/day; Q3, 4.5–9 mL/day; Q4, >9 mL/day; [f] categories of wine intake were as follow: Q1, none; Q2, <100 mL/day; Q3, 100–250 mL/day; Q4, >250 mL/day; [g] categories of beer intake were as follow: Q1, none; Q2, <100 mL/day; Q3, 100–300 mL/day; Q4, >300 mL/day.

4. Discussion

In this study, the relation between phenolic acid intake and hypertension was assessed in a cohort of adults living on a Mediterranean island. Dietary intake of phenolic acids in the highest quartile (roughly >400 mg/day) was inversely associated with hypertension; among the main classes and individual compounds investigated, hydroxybenzoic, and hydroxyphenylacetic acids showed a linear inverse association with having hypertension. When considering major dietary sources of phenolic acids, no specific food showed significant association with hypertension besides beer intake.

Observational studies exploring the association between phenolic acids and hypertension are scarce. In a cross-sectional study conducted in 550 adults living in Brazil the highest intake of phenolic acids was not associated with having hypertension, but the middle category of exposure (about 300 mg/day) showed an inverse association [16]. Another cross-sectional study conducted on 2618 Iranian adults showed no association between phenolic acid intake and hypertension as a component of metabolic syndrome; however, intake reported in this study was particularly low compared to the others (median intake of the highest category of exposure was 70.7 mg/day), suggesting that a lower number of food sources might have been used and, thus, data might not be reliable [15]. In contrast, a cross-sectional study conducted on 8821 men and women living in Poland showed that individuals with higher intake of phenolic acids were less likely to have impaired blood pressure (as component of the metabolic syndrome) compared to those with lower intake [17]; interestingly, a prospective study conducted on a subgroup of individuals belonging to the same cohort (those without hypertension on baseline), confirmed the previous findings, despite the results being significant only among women [18].

Among others, the compounds proposed to be responsible for beneficial effects on endothelium are phenolic acids [30,31]. Phenolic acids have been reported to improve endothelial health, and protect against atherothrombosis or atherosclerotic lesion development through attenuation of oxidative stress [32]. The hypotensive properties of phenolic acids have been hypothesized to depend on several mechanisms, such as a NO-mediated vasodilatatory effect, the attenuation of oxidative stress (reactive oxygen species) by reducing NAD(P)H-dependent (nicotinamide adenine dinucleotide phosphate) super-oxide production, and the interaction with the renin–angiotensin aldosterone system by inhibiting angiotensin-converting enzyme activity [33,34]. These compounds may also exert antioxidant effects by scavenging or neutralizing reactive oxidant species and building

endogenous antioxidant defenses by down-regulating inflammatory genes in endothelial cells and macrophages [35].

In the present study, only hydroxycinnamic and hydroxyphenenlacetic acids were associated with hypertension. It may be hypothesized that the retrieved associations between consumption of certain foods and blood pressure may be mediated, at least in part, by their content in phenolic acids. However, among major sources of hydroxycinnamic acids, coffee did not show an independent association with blood pressure levels, despite recent evidence showed a decreased risk of hypertension in cohort studies [36,37]. In contrast, a significant inverse association between beer consumption (which was the major source of hydroxyphenenlacetic acids) and hypertension was found; despite heavy alcohol intake being associated with a higher risk of hypertension [38], a large amount of scientific literature agrees that moderate consumption of alcoholic beverages may exert beneficial effects on human health [39,40]. Meta-analyses of prospective cohort studies showed decreased risk of mortality of cardiovascular causes for moderate consumption of alcohol [41]. The significant reduction in vascular risk with a J-shaped relation associated with wine and beer consumption indicated a comparable protecting effect of either beverage potentially due to their common components, such as polyphenols. Around 30% of polyphenols from beer are derived from hops and 70–80% originates from malt [42]; the structural classes of polyphenols in beer include phenols, and benzoic and cinnamic acid derivatives, including hydroxyphenylacetic acid, which was inversely associated with hypertension in the present study.

Some of the food groups investigated in this study are characteristic of healthy dietary patterns, such as the Mediterranean diet. The effect of nut and olive oil consumption on blood pressure has been investigated within the context of healthy diets showing significant results [43–48]. Clinical studies showed that moderate consumption of wine and beer as part of the Mediterranean diet had beneficial effects toward biomarkers of hypertension [49,50], especially among fast ethanol metabolizers [51]. Recent evidence suggests that the beneficial effects of the Mediterranean diet may depend on their content in polyphenols [52–55]. The Mediterranean dietary pattern has been associated with lower risk of cardio-metabolic disorders [56–61]. Previous results from this cohort showed a significant inverse association between higher adherence to this dietary pattern and hypertension [62]; however, another study showed that phenolic acid intake was associated in a non-linear manner to high adherence to the Mediterranean diet, suggesting that certain food sources of such polyphenol group not included in the classical paradigm of key features of the Mediterranean diet may provide beneficial action toward cardiovascular health [63]. Additionally, other studies conducted on a similar population (a sample recruited in the same southern Italian area) reported that food groups not included in the Mediterranean diet (i.e., coffee and beer) were associated with better metabolic health [64–66]. Overall, adherence to the Mediterranean dietary pattern might be an optimal choice for preventing non-communicable chronic diseases; moreover, the inclusion of certain foods generally not contemplated in this dietary pattern (such as coffee or beer) may improve the aforementioned potential benefits [67].

The findings of the present study should be considered in light of some limitations. The study design was cross-sectional, thus, we estimated the strength of association between the variables of interest and hypertension, but we are unable to define causal relation. Such a study design may be associated with reverse causation, with individuals affected by disease being less likely to have a higher intake of certain foods (i.e., coffee). However, the study was rather focused on phenolic acids, which may have several dietary sources and showed more consistent results than individual foods. Second, questions of FFQ on nut consumption did not refer to salt content (i.e., salted peanuts), thus, we are unaware whether nuts consumed were actually salted. However, in our analyses we adjusted for micronutrients related to salt intake in order to limit the potential confounding effect; moreover, whether confounding exists, it would weaken, rather than strengthen, the association between nut consumption and hypertension.

5. Conclusions

In conclusion, the findings of this study suggest that dietary phenolic acids may be inversely associated with hypertension, irrespective of their dietary source. Thus, phenolic acid content of the diet may be responsible for the observed beneficial effects on blood pressure of certain foods. Dietary intervention programs promoting increased intake of phenolic acids through higher consumption of functional foods are needed to confirm the promising findings obtained from this study. More in-depth laboratory studies are also needed to corroborate the exact mechanisms of action of such foods in order to better understand which compounds improve endothelial health and may affect the risk of hypertension.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/10/1069/s1, Supplementary material (Table S1. Dietary intake of major micro- and macro-nutrients by quartiles of dietary phenolic acids in the MEAL study sample (n = 1936); Table S2. Association between total, main classes, and individual phenolic acids and hypertension, separately for men and women).

Acknowledgments: We would like to thank all reviewers for their critical comments.

Author Contributions: J.G. conceived and designed the study, and performed the analysis, interpretation, and manuscript drafting; J.G. and D.S. managed the data; D.S. and I.B. performed the analysis; S.M. and M.L.W. critically revised the paper; and M.M. provided insights on data interpretation and critically revised the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Maghsoudi, Z.; Ghiasvand, R.; Salehi-Abargouei, A. Empirically derived dietary patterns and incident type 2 diabetes mellitus: A systematic review and meta-analysis on prospective observational studies. *Public Health Nutr.* **2016**, *19*, 230–241. [CrossRef] [PubMed]
2. Rezagholizadeh, F.; Djafarian, K.; Khosravi, S.; Shab-Bidar, S. A posteriori healthy dietary patterns may decrease the risk of central obesity: Findings from a systematic review and meta-analysis. *Nutr. Res.* **2017**, *41*, 1–13. [CrossRef] [PubMed]
3. Wang, C.J.; Shen, Y.X.; Liu, Y. Empirically derived dietary patterns and hypertension likelihood: A meta-analysis. *Kidney Blood Press Res.* **2016**, *41*, 570–581. [CrossRef] [PubMed]
4. Beidokhti, M.N.; Jager, A.K. Review of antidiabetic fruits, vegetables, beverages, oils and spices commonly consumed in the diet. *J. Ethnopharmacol.* **2017**, *201*, 26–41. [CrossRef] [PubMed]
5. Grosso, G.; Godos, J.; Galvano, F.; Giovannucci, E.L. Coffee, caffeine, and health outcomes: An umbrella review. *Annu. Rev. Nutr.* **2017**, *37*, 131–156. [CrossRef] [PubMed]
6. Schwingshackl, L.; Hoffmann, G.; Missbach, B.; Stelmach-Mardas, M.; Boeing, H. An umbrella review of nuts intake and risk of cardiovascualr disease. *Curr. Pharm. Des.* **2017**, *23*, 1016–1027. [CrossRef] [PubMed]
7. De Gaetano, G.; Costanzo, S.; Di Castelnuovo, A.; Badimon, L.; Bejko, D.; Alkerwi, A.; Chiva-Blanch, G.; Estruch, R.; La Vecchia, C.; Panico, S.; et al. Effects of moderate beer consumption on health and disease: A consensus document. *Nutr. Metab. Cardiovasc. Dis.* **2016**, *26*, 443–467. [CrossRef] [PubMed]
8. Grosso, G.; Yang, J.; Marventano, S.; Micek, A.; Galvano, F.; Kales, S.N. Nut consumption on all-cause, cardiovascular, and cancer mortality risk: A systematic review and meta-analysis of epidemiologic studies. *Am. J. Clin. Nutr.* **2015**, *101*, 783–793. [CrossRef] [PubMed]
9. Grosso, G.; Micek, A.; Godos, J.; Sciacca, S.; Pajak, A.; Martinez-Gonzalez, M.A.; Giovannucci, E.L.; Galvano, F. Coffee consumption and risk of all-cause, cardiovascular, and cancer mortality in smokers and non-smokers: A dose-response meta-analysis. *Eur. J. Epidemiol.* **2016**, *31*, 1191–1205. [CrossRef] [PubMed]
10. Costanzo, S.; Di Castelnuovo, A.; Donati, M.B.; Iacoviello, L.; de Gaetano, G. Alcohol consumption and mortality in patients with cardiovascular disease: A meta-analysis. *J. Am. Coll. Cardiol.* **2010**, *55*, 1339–1347. [CrossRef] [PubMed]
11. Rienks, J.; Barbaresko, J.; Nothlings, U. Association of polyphenol biomarkers with cardiovascular disease and mortality risk: A systematic review and meta-analysis of observational studies. *Nutrients* **2017**, *9*, 415. [CrossRef] [PubMed]

12. Grosso, G.; Micek, A.; Godos, J.; Pajak, A.; Sciacca, S.; Galvano, F.; Giovannucci, E.L. Dietary flavonoid and lignan intake and mortality in prospective cohort studies: Systematic review and dose-response meta-analysis. *Am. J. Epidemiol.* **2017**, 1–13. [CrossRef] [PubMed]
13. Del Rio, D.; Costa, L.G.; Lean, M.E.; Crozier, A. Polyphenols and health: What compounds are involved? *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 1–6. [CrossRef] [PubMed]
14. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox Signal.* **2013**, *18*, 1818–1892. [CrossRef] [PubMed]
15. Sohrab, G.; Hosseinpour-Niazi, S.; Hejazi, J.; Yuzbashian, E.; Mirmiran, P.; Azizi, F. Dietary polyphenols and metabolic syndrome among Iranian adults. *Int. J. Food Sci. Nutr.* **2013**, *64*, 661–667. [CrossRef] [PubMed]
16. Miranda, A.M.; Steluti, J.; Fisberg, R.M.; Marchioni, D.M. Association between polyphenol intake and hypertension in adults and older adults: A population-based study in Brazil. *PLoS ONE* **2016**, *11*, e0165791. [CrossRef] [PubMed]
17. Grosso, G.; Stepaniak, U.; Micek, A.; Stefler, D.; Bobak, M.; Pajak, A. Dietary polyphenols are inversely associated with metabolic syndrome in polish adults of the hapiee study. *Eur. J. Nutr.* **2017**, *56*, 1409–1420. [CrossRef] [PubMed]
18. Grosso, G.; Stepaniak, U.; Micek, A.; Kozela, M.; Stefler, D.; Bobak, M.; Pajak, A. Dietary polyphenol intake and risk of hypertension in the polish arm of the hapiee study. *Eur. J. Nutr.* **2017**. [CrossRef] [PubMed]
19. Grosso, G.; Marventano, S.; D'Urso, M.; Mistretta, A.; Galvano, F. The Mediterranean healthy eating, ageing, and lifestyle (meal) study: Rationale and study design. *Int. J. Food Sci. Nutr.* **2017**, *68*, 577–586. [CrossRef] [PubMed]
20. Mistretta, A.; Marventano, S.; Platania, A.; Godos, J.; Galvano, F.; Grosso, G. Metabolic profile of the Mediterranean healthy eating, lifestyle and aging (meal) study cohort. *Mediterr. J. Nutr. Metab.* **2017**, *10*, 131–140. [CrossRef]
21. Craig, C.L.; Marshall, A.L.; Sjostrom, M.; Bauman, A.E.; Booth, M.L.; Ainsworth, B.E.; Pratt, M.; Ekelund, U.; Yngve, A.; Sallis, J.F.; et al. International physical activity questionnaire: 12-country reliability and validity. *Med. Sci. Sports Exerc.* **2003**, *35*, 1381–1395. [CrossRef] [PubMed]
22. Buscemi, S.; Rosafio, G.; Vasto, S.; Massenti, F.M.; Grosso, G.; Galvano, F.; Rini, N.; Barile, A.M.; Maniaci, V.; Cosentino, L.; et al. Validation of a food frequency questionnaire for use in italian adults living in sicily. *Int. J. Food Sci. Nutr.* **2015**, *66*, 426–438. [CrossRef] [PubMed]
23. Marventano, S.; Mistretta, A.; Platania, A.; Galvano, F.; Grosso, G. Reliability and relative validity of a food frequency questionnaire for italian adults living in sicily, southern Italy. *Int. J. Food Sci. Nutr.* **2016**, *67*, 857–864. [CrossRef] [PubMed]
24. Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione. *Tabelle di Composizione Degli Alimenti*; Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione: Roma, Italy, 2009.
25. Godos, J.; Marventano, S.; Mistretta, A.; Galvano, F.; Grosso, G. Dietary sources of polyphenols in the Mediterranean healthy eating, aging and lifestyle (meal) study cohort. *Int. J. Food Sci. Nutr.* **2017**, *68*, 750–756. [CrossRef] [PubMed]
26. Neveu, V.; Perez-Jiménez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D.; et al. Phenol-explorer: An online comprehensive database on polyphenol contents in foods. *Database* **2010**. [CrossRef] [PubMed]
27. Rothwell, J.A.; Perez-Jimenez, J.; Neveu, V.; Medina-Remon, A.; M'Hiri, N.; Garcia-Lobato, P.; Manach, C.; Knox, C.; Eisner, R.; Wishart, D.S.; et al. Phenol-explorer 3.0: A major update of the phenol-explorer database to incorporate data on the effects of food processing on polyphenol content. *Database* **2013**, *2013*, bat070. [CrossRef] [PubMed]
28. Bognar, A. *Tables on Weight Yield of Food and Retention Factors of Food Constituents for the Calculation of Nutrient Composition of Cooked Foods (Dishes)*; Federal Research Centre for Nutrition: Karlsruhe, Germany, 2002.
29. Willett, W. Reproducibility and validity of food frequency questionnaire. In *Nutritional Epidemiology*, 2nd ed.; Press, O.U.: Norman, OK, USA, 1998.
30. Buscemi, S.; Marventano, S.; Antoci, M.; Cagnetti, A.; Castorina, G.; Galvano, F.; Marranzano, M.; Mistretta, A. Coffee and metabolic impairment: An updated review of epidemiological studies. *NFS J.* **2016**, *3*, 1–7. [CrossRef]

31. Bolling, B.W.; Chen, C.Y.; McKay, D.L.; Blumberg, J.B. Tree nut phytochemicals: Composition, antioxidant capacity, bioactivity, impact factors. A systematic review of almonds, brazils, cashews, hazelnuts, macadamias, pecans, pine nuts, pistachios and walnuts. *Nutr. Res. Rev.* **2011**, *24*, 244–275. [CrossRef] [PubMed]
32. Barbour, J.A.; Howe, P.R.; Buckley, J.D.; Bryan, J.; Coates, A.M. Nut consumption for vascular health and cognitive function. *Nutr. Res. Rev.* **2014**, *27*, 131–158. [CrossRef] [PubMed]
33. Zhao, Y.; Wang, J.; Ballevre, O.; Luo, H.; Zhang, W. Antihypertensive effects and mechanisms of chlorogenic acids. *Hypertens. Res.* **2012**, *35*, 370–374. [CrossRef] [PubMed]
34. Ochiai, R.; Sugiura, Y.; Otsuka, K.; Katsuragi, Y.; Hashiguchi, T. Coffee bean polyphenols ameliorate postprandial endothelial dysfunction in healthy male adults. *Int. J. Food Sci. Nutr.* **2015**, *66*, 350–354. [CrossRef] [PubMed]
35. Rahman, I.; Biswas, S.K.; Kirkham, P.A. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem. Pharmacol.* **2006**, *72*, 1439–1452. [CrossRef] [PubMed]
36. Marventano, S.; Salomone, F.; Godos, J.; Pluchinotta, F.; Del Rio, D.; Mistretta, A.; Grosso, G. Coffee and tea consumption in relation with non-alcoholic fatty liver and metabolic syndrome: A systematic review and meta-analysis of observational studies. *Clin. Nutr.* **2016**, *35*, 1269–1281. [CrossRef] [PubMed]
37. Grosso, G.; Micek, A.; Godos, J.; Pajak, A.; Sciacca, S.; Bes-Rastrollo, M.; Galvano, F.; Martinez-Gonzalez, M.A. Long-term coffee consumption is associated with decreased incidence of new-onset hypertension: A dose-response meta-analysis. *Nutrients* **2017**, *9*, 890. [CrossRef] [PubMed]
38. Briasoulis, A.; Agarwal, V.; Messerli, F.H. Alcohol consumption and the risk of hypertension in men and women: A systematic review and meta-analysis. *J. Clin. Hypertens.* **2012**, *14*, 792–798. [CrossRef] [PubMed]
39. Poli, A.; Marangoni, F.; Avogaro, A.; Barba, G.; Bellentani, S.; Bucci, M.; Cambieri, R.; Catapano, A.L.; Costanzo, S.; Cricelli, C.; et al. Moderate alcohol use and health: A consensus document. *Nutr. Metab. Cardiovasc. Dis.* **2013**, *23*, 487–504. [CrossRef] [PubMed]
40. Giacosa, A.; Adam-Blondon, A.F.; Baer-Sinnott, S.; Barale, R.; Bavaresco, L.; Di Gaspero, G.; Dugo, L.; Ellison, R.C.; Gerbi, V.; Gifford, D.; et al. Alcohol and wine in relation to cancer and other diseases. *Eur. J. Cancer Prev.* **2012**, *21*, 103–108. [CrossRef] [PubMed]
41. Di Castelnuovo, A.; Costanzo, S.; Bagnardi, V.; Donati, M.B.; Iacoviello, L.; de Gaetano, G. Alcohol dosing and total mortality in men and women: An updated meta-analysis of 34 prospective studies. *Arch. Intern. Med.* **2006**, *166*, 2437–2445. [CrossRef] [PubMed]
42. Arranz, S.; Chiva-Blanch, G.; Valderas-Martinez, P.; Medina-Remon, A.; Lamuela-Raventos, R.M.; Estruch, R. Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. *Nutrients* **2012**, *4*, 759–781. [CrossRef] [PubMed]
43. Adamsson, V.; Reumark, A.; Fredriksson, I.B.; Hammarstrom, E.; Vessby, B.; Johansson, G.; Riserus, U. Effects of a healthy nordic diet on cardiovascular risk factors in hypercholesterolaemic subjects: A randomized controlled trial (nordiet). *J. Intern. Med.* **2011**, *269*, 150–159. [CrossRef] [PubMed]
44. Esposito, K.; Marfella, R.; Ciotola, M.; Di Palo, C.; Giugliano, F.; Giugliano, G.; D'Armiento, M.; D'Andrea, F.; Giugliano, D. Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: A randomized trial. *JAMA* **2004**, *292*, 1440–1446. [CrossRef] [PubMed]
45. Thomazella, M.C.; Goes, M.F.; Andrade, C.R.; Debbas, V.; Barbeiro, D.F.; Correia, R.L.; Marie, S.K.; Cardounel, A.J.; daLuz, P.L.; Laurindo, F.R. Effects of high adherence to Mediterranean or low-fat diets in medicated secondary prevention patients. *Am. J. Cardiol.* **2011**, *108*, 1523–1529. [CrossRef] [PubMed]
46. Toledo, E.; Hu, F.B.; Estruch, R.; Buil-Cosiales, P.; Corella, D.; Salas-Salvado, J.; Covas, M.I.; Aros, F.; Gomez-Gracia, E.; Fiol, M.; et al. Effect of the Mediterranean diet on blood pressure in the predimed trial: Results from a randomized controlled trial. *BMC Med.* **2013**, *11*, 207. [CrossRef] [PubMed]
47. Storniolo, C.E.; Casillas, R.; Bullo, M.; Castaner, O.; Ros, E.; Saez, G.T.; Toledo, E.; Estruch, R.; Ruiz-Gutierrez, V.; Fito, M.; et al. A Mediterranean diet supplemented with extra virgin olive oil or nuts improves endothelial markers involved in blood pressure control in hypertensive women. *Eur. J. Nutr.* **2017**, *56*, 89–97. [CrossRef] [PubMed]
48. Grosso, G.; Estruch, R. Nut consumption and age-related disease. *Maturitas* **2016**, *84*, 11–16. [CrossRef] [PubMed]

49. Tresserra-Rimbau, A.; Medina-Remon, A.; Lamuela-Raventos, R.M.; Bullo, M.; Salas-Salvado, J.; Corella, D.; Fito, M.; Gea, A.; Gomez-Gracia, E.; Lapetra, J.; et al. Moderate red wine consumption is associated with a lower prevalence of the metabolic syndrome in the predimed population. *Br. J. Nutr.* **2015**, *113* (Suppl. S2), S121–S130. [CrossRef] [PubMed]
50. Giacosa, A.; Barale, R.; Bavaresco, L.; Faliva, M.A.; Gerbi, V.; La Vecchia, C.; Negri, E.; Opizzi, A.; Perna, S.; Pezzotti, M.; et al. Mediterranean way of drinking and longevity. *Crit. Rev. Food Sci. Nutr.* **2016**, *56*, 635–640. [CrossRef] [PubMed]
51. Gepner, Y.; Henkin, Y.; Schwarzfuchs, D.; Golan, R.; Durst, R.; Shelef, I.; Harman-Boehm, I.; Spitzen, S.; Witkow, S.; Novack, L.; et al. Differential effect of initiating moderate red wine consumption on 24-h blood pressure by alcohol dehydrogenase genotypes: Randomized trial in type 2 diabetes. *Am. J. Hypertens.* **2016**, *29*, 476–483. [CrossRef] [PubMed]
52. Bawaked, R.A.; Schroder, H.; Ribas-Barba, L.; Cardenas, G.; Pena-Quintana, L.; Perez-Rodrigo, C.; Fito, M.; Serra-Majem, L. Dietary flavonoids of spanish youth: Intakes, sources, and association with the Mediterranean diet. *PeerJ* **2017**, *5*, e3304. [CrossRef] [PubMed]
53. Bonaccio, M.; Pounis, G.; Cerletti, C.; Donati, M.B.; Iacoviello, L.; de Gaetano, G.; Investigators, M.-S.S. Mediterranean diet, dietary polyphenols and low grade inflammation: Results from the moli-sani study. *Br. J. Clin. Pharmacol.* **2017**, *83*, 107–113. [CrossRef] [PubMed]
54. Medina-Remon, A.; Casas, R.; Tressserra-Rimbau, A.; Ros, E.; Martinez-Gonzalez, M.A.; Fito, M.; Corella, D.; Salas-Salvado, J.; Lamuela-Raventos, R.M.; Estruch, R.; et al. Polyphenol intake from a Mediterranean diet decreases inflammatory biomarkers related to atherosclerosis: A substudy of the predimed trial. *Br. J. Clin. Pharmacol.* **2017**, *83*, 114–128. [CrossRef] [PubMed]
55. Zamora-Ros, R.; Knaze, V.; Lujan-Barroso, L.; Romieu, I.; Scalbert, A.; Slimani, N.; Hjartaker, A.; Engeset, D.; Skeie, G.; Overvad, K.; et al. Differences in dietary intakes, food sources and determinants of total flavonoids between Mediterranean and non-Mediterranean countries participating in the european prospective investigation into cancer and nutrition (epic) study. *Br. J. Nutr.* **2013**, *109*, 1498–1507. [CrossRef] [PubMed]
56. Godos, J.; Federico, A.; Dallio, M.; Scazzina, F. Mediterranean diet and nonalcoholic fatty liver disease: Molecular mechanisms of protection. *Int. J. Food Sci. Nutr.* **2016**, *68*, 18–27. [CrossRef] [PubMed]
57. Godos, J.; Zappala, G.; Bernardini, S.; Giambini, I.; Bes-Rastrollo, M.; Martinez-Gonzalez, M. Adherence to the Mediterranean diet is inversely associated with metabolic syndrome occurrence: A meta-analysis of observational studies. *Int. J. Food Sci. Nutr.* **2017**, *68*, 138–148. [CrossRef] [PubMed]
58. Grosso, G.; Mistretta, A.; Marventano, S.; Purrello, A.; Vitaglione, P.; Calabrese, G.; Drago, F.; Galvano, F. Beneficial effects of the Mediterranean diet on metabolic syndrome. *Curr. Pharm. Des.* **2014**, *20*, 5039–5044. [CrossRef] [PubMed]
59. Grosso, G.; Marventano, S.; Yang, J.; Micek, A.; Pajak, A.; Scalfi, L.; Galvano, F.; Kales, S.N. A comprehensive meta-analysis on evidence of Mediterranean diet and cardiovascular disease: Are individual components equal? *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 3218–3232. [CrossRef] [PubMed]
60. Buscemi, S.; Marventano, S.; Castellano, S.; Nolfo, F.; Rametta, S.; Giorgianni, G.; Matalone, M.; Marranzano, M.; Mistretta, A. Role of anthropometric factors, self-perception, and diet on weight misperception among young adolescents: A cross-sectional study. *Eat. Weight Disord.* **2016**. [CrossRef] [PubMed]
61. Mistretta, A.; Marventano, S.; Antoci, M.; Cagnetti, A.; Giogianni, G.; Nolfo, F.; Rametta, S.; Pecora, G.; Marranzano, M. Mediterranean diet adherence and body composition among southern Italian adolescents. *Obes. Res. Clin. Pract.* **2017**, *11*, 215–226. [CrossRef] [PubMed]
62. La Verde, M.; Mulè, S.; Zappalà, G.; Privitera, G.; Maugeri, G.; Pecora, F.; Marranzano, M. Higher adherence to the Mediterranean diet is inversely associated with having hypertension: Is low salt intake a mediating factor? *Int. J. Food Sci. Nutr.* **2017**, *14*, 1–10. [CrossRef] [PubMed]
63. Godos, J.; Rapisarda, G.; Marventano, S.; Galvano, F.; Mistretta, A.; Grosso, G. Association between polyphenol intake and adherence to the Mediterranean diet in sicily, southern Italy. *NFS J.* **2017**, *8*, 1–7. [CrossRef]
64. Grosso, G.; Pajak, A.; Mistretta, A.; Marventano, S.; Raciti, T.; Buscemi, S.; Drago, F.; Scalfi, L.; Galvano, F. Protective role of the Mediterranean diet on several cardiovascular risk factors: Evidence from sicily, southern Italy. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 370–377. [CrossRef] [PubMed]

65. Grosso, G.; Marventano, S.; Giorgianni, G.; Raciti, T.; Galvano, F.; Mistretta, A. Mediterranean diet adherence rates in sicily, southern Italy. *Public Health Nutr.* **2014**, *17*, 2001–2009. [CrossRef] [PubMed]
66. Grosso, G.; Marventano, S.; Galvano, F.; Pajak, A.; Mistretta, A. Factors associated with metabolic syndrome in a Mediterranean population: Role of caffeinated beverages. *J. Epidemiol.* **2014**, *24*, 327–333. [CrossRef] [PubMed]
67. Giacosa, A.; Barale, R.; Bavaresco, L.; Gatenby, P.; Gerbi, V.; Janssens, J.; Johnston, B.; Kas, K.; La Vecchia, C.; Mainguet, P.; et al. Cancer prevention in europe: The Mediterranean diet as a protective choice. *Eur. J. Cancer Prev.* **2013**, *22*, 90–95. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
Tel. +41 61 683 77 34
Fax +41 61 302 89 18
www.mdpi.com

Nutrients Editorial Office
E-mail: nutrients@mdpi.com
www.mdpi.com/journal/nutrients

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland

Tel: +41 61 683 77 34
Fax: +41 61 302 89 18

www.mdpi.com

ISBN 978-3-03897-142-9