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Dietary Intakes and Metabolic Disorders

Edited by
Gemma Chiva-Blanch and Montserrat Cofán

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Contents

Preface to "Dietary Intakes and Metabolic Disorders"

Metabolic syndrome is defined by a cluster of inter-related cardiovascular risk factors (hyperglycemia, hypertension, dyslipidemia, insulin resistance, and central adiposity) linked to chronic, systemic, and low-grade inflammation, which severely increases the risk of type II diabetes and cardiovascular disease. Before, within, and hopefully after the COVID pandemics, metabolic diseases are still one of the major silent pandemics worldwide, causing at least more than 2.5 million deaths annually. A growing body of evidence generated in recent decades has shown that not only the energy balance but also the macro-, micro-, and non-nutrient composition of the diet may influence the onset and progression of metabolic syndrome. However, further research is required to increase our understanding of the contribution of diet to the prevention of metabolic syndrome. We propose this Special Issue, entitled "Dietary Intakes and Metabolic Syndrome", with the aim of gathering original research manuscripts and reviews dealing with the relationships between diet as a whole and metabolic-syndrome-related diseases, as well as with the effects of specific nutrients or non-nutritional bioactive compounds on this metabolic condition and related pathologic features. In the end, the Special Issue covers important issues such as nutrition and bioenergetics, the legacy effect in nutrition, the effects of specific foods and dietary patterns on type II diabetes mellitus, or the always-controversial omega 3 fatty acids. We hope that this Special Issue will be helpful for healthcare providers and researchers in nutrition and/or cardio- and metabolic diseases, to reflect on, in more depth, the complex system of nutrition and metabolic syndrome. Finally, we would like to thank all of the authors and reviewers for their contributions, in addition to the MDPI staff and the Editors-in-Chief of Nutrients for their kind assistance in the process. We sincerely hope that you enjoy reading this Special Issue.

Gemma Chiva-Blanch and Montserrat Cofán
Editors

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Review

Nutrition, Bioenergetics, and Metabolic Syndrome

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Abstract: According to the World Health Organization (WHO), the global nutrition report shows that whilst part of the world's population starves, the other part suffers from obesity and associated complications. A balanced diet counterparts these extreme conditions with the proper proportion, composition, quantity, and presence of macronutrients, micronutrients, and bioactive compounds. However, little is known on the way these components exert any influence on our health. These nutrients aiming to feed our bodies, our tissues, and our cells, first need to reach mitochondria, where they are decomposed into CO_2 and H_2O to obtain energy. Mitochondria are the powerhouse of the cell and mainly responsible for nutrients metabolism, but they are also the main source of oxidative stress and cell death by apoptosis. Unappropriated nutrients may support mitochondrial to become the Trojan horse in the cell. This review aims to provide an approach to the role that some nutrients exert on mitochondria as a major contributor to high prevalent Western conditions including metabolic syndrome (MetS), a constellation of pathologic conditions which promotes type II diabetes and cardiovascular risk. Clinical and experimental data extracted from in vitro animal and cell models further demonstrated in patients, support the idea that a balanced diet, in a healthy lifestyle context, promotes proper bioenergetic and mitochondrial function, becoming the best medicine to prevent the onset and progression of MetS. Any advance in the prevention and management of these prevalent complications help to face these challenging global health problems, by ameliorating the quality of life of patients and reducing the associated sociosanitary burden.

Keywords: mitochondria; metabolic syndrome; mitochondrial dysfunction; balanced diet; nutrients; lifestyle

1. Introduction

In the last decades, global dietary patterns have experienced a transition towards increasingly Westernized and less healthier diets. This trend is linked to the substantial increase in the prevalence of non-communicable diseases (NCDs) such as type II diabetes (T2D) or cardiovascular disease (CVD) [1]. During the last 40 years, the global prevalence of T2D has almost doubled, from 4.7% in 1980 to 8.5% in 2014. Likewise, the number of deaths attributed to CVD (mainly coronary heart disease, stroke, and rheumatic heart disease) has increased from 14.4 million in 1990 to 17.5 million, being the number 1 cause of death globally [2].

The metabolic syndrome (MetS), a cluster of pathologic conditions that raise the risk of CVD and T2D [3], has been emerged as a health problem in modern society, associated with enormous social, personal and economic burden in both the developing and developed world [4]. Currently, MetS is calculated to affect 25% of the global population [5] and all the epidemiological studies have

demonstrated its prevalence increases with age [6]. Additionally, the growing age of the population in first world societies will increase such trends in coming years.

International food organizations have traditionally focused on food security and micronutrient deficiency. However, diet-related health burdens contributing to the above-mentioned diseases are now surpassing those due to undernutrition in nearly every region of the world. Therefore, the establishment of healthy dietary patterns is a global priority to reduce the onset of these diseases [7].

There are plenty of studies establishing healthy dietary patterns [1]. However, scarce knowledge is available on the way these nutrients underscore or promote our health. First, because there is a wide panoply of nutrients and, second, because each one of them can trigger a multitude of effects, organ, tissue, cell, and even molecule specific. However, a universal fact is that all of them need to be processed into small subunits and metabolized by mitochondria to obtain energy. Thus, mitochondrial function is key to understand dietary effects in our organism and, at the same time, understanding how dietary patterns and nutrients influence mitochondrial function is essential to establish causal relationships between food and health.

The diet's impact on mitochondrial function has been widely studied due to its role as a powerhouse of the cell and its involvement in nutrients' metabolism. In fact, mitochondria are critical in the maintenance of metabolic flexibility, efficient switches in metabolism depending on the environmental demand (feeding/fasting cycles) [8]. Moreover, also the intake amount (p.e. high-fat and/or sugars diet) conditionate mitochondrial function [9]. Additionally, micronutrients (the ~40 essential vitamins, minerals, fatty acids, and amino acids) and bioactive compounds, must be considered in a balanced diet to get an optimal cell–mitochondrial–metabolic axis function [10].

These dietary considerations are closely related to the manifestation of the most common NCDs [11], including MetS [12], where the mitochondrial dys(function) can be considered a common hallmark.

Consequently, the aim of this work is to review the role of some nutrients, easily incorporable in a balanced diet, over mitochondrial (dys)function and its impact on MetS. For this purpose, we herein have reviewed data extracted from experimental and clinical studies published in PubMed and Google Scholar database, from inception to July 2020, to document the principal examples of nutrients' effect on mitochondrial activity and their influence on MetS.

2. A Healthy Diet

Diet is the most significant single risk factor for disability and premature death [13]. Dietary choices contribute to the development of pathologic conditions such as hypertension, hypercholesterolemia, overweight/obesity, and inflammation, which in turn increase the risk for associated diseases, including CVD or T2D. Several studies have demonstrated the causal link between the global shift towards Westernized dietary patterns, characterized by high intakes of red and processed meat, pre-packaged foods, sweets, and refined grains, and lacking in fruits and vegetables, whole-grains, fish, and nuts, and the increased prevalence of these diet-related chronic diseases in the last decades [1,14].

Dietary changes recommended by the World Health Organization (WHO) include balancing energy intake, limiting saturated and trans fats, and shifting toward unsaturated fats consumption, increasing intake of fruits and vegetables, and limiting the consumption of sugar and salt. These dietary patterns are naturally occurring in certain world regions, as it is the case of the Mediterranean diet (MedDiet) and traditional Asian diets such as Korean, Chinese, and Japanese diets. Both the MedDiet (rich in fish, monounsaturated fats from olive oil, fruits, vegetables, whole grains, and legumes/nuts) and traditional Asian diets (composed of rice and other whole grains, fermented food, soy, fruits and vegetables, fish and seafood) [1] have moderate to strong evidence for preventing T2D and MetS, decreasing cancer and CVD incidence and mortality, decreasing overall mortality, and treating obesity [15–18]. Moreover, healthy dietary patterns have also been developed based on nutrient intake studies and their outcomes, as it is the case of DASH (Dietary Approaches to Stop Hypertension) [19] and MIND (Mediterranean-DASH Intervention for Neurodegenerative Delay) diets [20], among others.

At the molecular level, a healthy diet is characterized by the consumption of appropriate proportions of macronutrients (carbohydrates, proteins, and fats) to support energetic and physiologic functions, sufficient micronutrients (vitamins and mineral) and hydration, to meet the physiologic needs of the body [1]. Additionally, there is increasing evidence of the beneficial role of bioactive compounds, such as polyphenols or lycopene, in a healthy diet to prevent diseases. These molecules, generally natural and non-nutritive compounds, can be found in small quantities in plants and lipid-rich foods, and are essential to fight age-related and chronic diseases [21].

3. Metabolic Syndrome (MetS)

The MetS is not a disease per se, but a constellation of pathologic conditions that raises the risk of CVD by 1.5–2.5 fold [22] and T2D by 3–20 fold, depending on the numbers of pathologic factors [23]. To be diagnosed with MetS, at least three of the five deregulations shown in Table 1 must be present in the patient [24].

Table 1. Risk factors associated with metabolic syndrome and definition of the parameters for its diagnosis.

Risk Factor	Parameters	Definition (Male/Female)
Central obesity	Waist circumference [1]	America: ≥102 cm/≥88 cm Asia: ≥90 cm/≥80 cm Europe: ≥94 cm/≥80 cm Africa: ≥94 cm/≥80 cm Middle East: ≥94 cm/≥80 cm
Hypertriglyceridemia	Triglyceride concentrations	≥150 mg/dL
Low HDL-cholesterol levels	HDL-cholesterol levels	≤40 mg/dL/<50 mg/dL
Hypertension	Systolic/diastolic blood pressure	≥130/85 mm Hg
Hyperglycemia	Fasting plasma glucose	≥100 mg/dL

[1] The definition of this measure varies among regions. HDL = High density lipoprotein.

The determining factors for the development of MetS are, in order of relevance/influence: weight, genetics, aging, and lifestyle [25]. Based on that, risk factors associated with MetS can be reduced by the introduction of a balanced-diet and some regular exercise [26].

4. Mitochondria in Health and Disease

Mitochondria are unique structures in the cell, originally alpha-proteobacterium phagocyted by a eukaryotic progenitor [27]. Since then, proper mitochondrial activity is critical for the survival of the eukaryotic cell. In fact, mitochondrial deregulation has been described as a hallmark underlying several complex diseases, since mitochondrial dysfunction can trigger, by its own, oxidative stress, bioenergetic failure, inflammation, protein accumulation, and cell death [28], common aspects in metabolic disorders, such as MetS (Figure 1). That is why the nature of mitochondrial disorders underlies the numerous roles that mitochondria play.

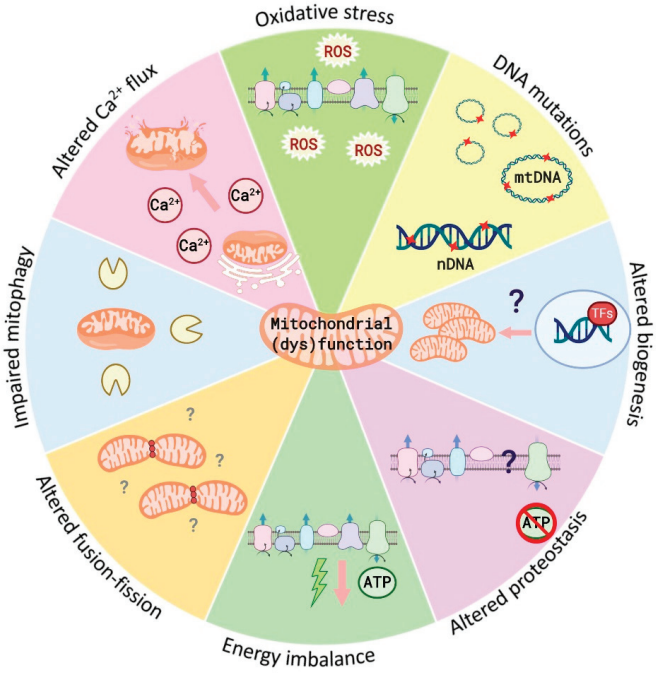


Figure 1. Mitochondrial (dys)function factors. Mitochondrial dysfunction is characterized by the combination of some of the processes described above and plays a central role in several diseases, such as metabolic syndrome. All these processes are closely related, usually affecting one each other and rarely having isolated effects. *Oxidative stress* leads to the accumulation of reactive oxygen species (ROS). In turn, high amount of ROS might trigger *DNA mutations.* Mitochondrial (dys)function can also be associated to *reduced mitochondrial biogenesis* through the decreased expression of mitochondrial genes. Moreover, *altered proteostasis* (biogenesis, folding, trafficking, and degradation of mitochondrial proteins) is common. Another frequent consequence is an *energy imbalance* limiting the ATP supply. Furthermore, mitochondrial (dys)function may condition mitochondrial *fusion-fission* processes compromising mitochondrial renewal and material exchange. Similarly, *impaired mitophagy* triggers the accumulation of non-functional mitochondria. Finally, mitochondrial *altered* Ca^{2+} *flux and buffering*, disrupt communication with other organelles such as endoplasmic reticulum and, in case of calcium release into the cytoplasm, the potential trigger of cell death.

4.1. Mitochondrial (Dys)Functions

The best-known mitochondrial function is energy production, through the generation of ATP molecules, via oxidative phosphorylation. The mitochondrial matrix houses the Krebs cycle (KC) enzymes, important producers of electron donors such as NADH and $FADH_2$. These molecules bring electrons to the mitochondrial respiratory chain (MRC), formed by four complexes (I-IV), that pump protons from the mitochondrial matrix into the inner membrane space through sequential redox reactions that finally reduce O_2 into H_2O at complex IV and, the complex V or ATP synthase, uses the accumulated proto-gradient energy to phosphorylate ADP into ATP (chemiosmotic theory of Mitchell).

One of the major cell producers of NADH is the oxidation of fatty acids (FA) to acyl-CoA, a process called β-oxidation, which takes place in the mitochondrial matrix. The acetyl-CoA resulting from this process can enter the KC and get oxidized, coupled to the production of reducing power [29]. Furthermore, alterations in these metabolic routes have also been widely described in cell and animal

models, as well as in patients suffering the most prevalent NCDs such as heart failure, T2D [30], or MetS [31,32].

Secondarily, these redox reactions generate reactive oxygen species (ROS) (Figure 2). Low ROS increase can trigger healthy physiological responses by activating complex transcriptional cascades of stress resistance, cell proliferation, and differentiation pathways [33]. However, when cells overproduce ROS, those can trigger pathologic consequences by promoting damage of cellular compounds (in sugars, lipids, proteins, or nucleic acids) [34,35], thus impairing cell proliferation, differentiation, and apoptosis, among others [36]. Additionally, mitochondria can trigger or amplify by their own cell death signals triggered by an alternative source of damage. Altogether, these effects may have a deep impact in mitochondrial deregulation and derived diseases, including MetS [37].

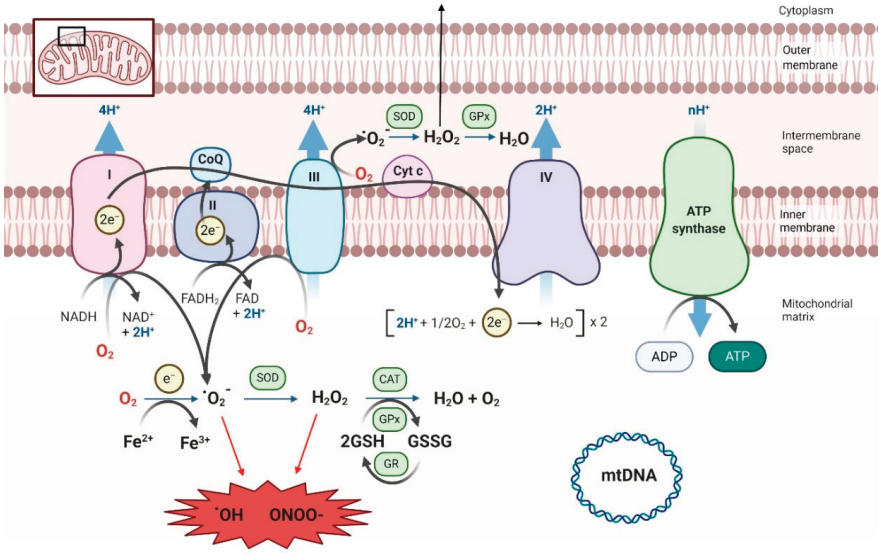


Figure 2. Main sources of mitochondrial reactive oxygen species (ROS). Mitochondrial respiratory chain (MRC) is composed of four enzyme complexes (I–IV). Electrons donated from NADH and $FADH_2$ in the Krebs cycle are transferred at complex I and II respectively, and consecutively to complex III and IV. This electrons transfer is coupled with protons transport across the inner membrane, generating a proton-gradient, allowing ADP phosphorylation through ATP synthase (complex V). Oxygen metabolism generates superoxide anion ($\cdot 0_2^-$), which in turn is conversed into hydrogen peroxide (H_2O_2) by SOD (superoxide dismutase) and then converted into water thanks to the action of some of the antioxidant enzymatic defenses such as catalase (CAT) or the glutathione peroxidase (GPx) reductase (GPr) system. Excessive ROS production can oxidize proteins, lipids, or mitochondrial DNA (mtDNA), eventually leading to mitochondrial dysfunction and cell death.

To fight oxidative stress, understood as the imbalance between ROS production and antioxidants in favor of ROS species, cells have several molecules involved in the antioxidant defense system. The enzymatic barrier includes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx). Whilst other barriers, non-enzymatic antioxidants molecules, are vitamins E and C, glutathione (GSH), and various carotenoids and flavonoids [4], present in foods and beverages.

The proton leak generated in the MRC is mainly used to produce ATP but can also be used for thermogenesis purposes or to reduce the mitochondrial membrane potential, aiming to protect cells from oxidative stress [38]. The uncoupling proteins (UCPs) are a family of 5 proteins responsible for

uncoupling and widely distributed in specific tissues. In fact, several studies in rodents and patients demonstrated abnormal expression or activity of UCPs related to obesity [39] and T2D [40].

Interestingly, mitochondrial functions reach far beyond bioenergetics. Mitochondria are the key organelles in iron metabolism, involved in heme and Fe-S clusters synthesis [41], essential structures in oxygen transport, MRC, and DNA repair machinery.

Moreover, mitochondria regulate Ca^{2+} homeostasis, a key factor regulating aerobic metabolism and one of the apoptosis triggering factors [42,43]. Alterations in these pathways are linked to NCDs [44,45].

For all the above mentioned, mitochondria are key organelles for cell survival and adaptation to changing environmental conditions. Thus, for instance, high energy requirements activate mitochondrial biogenesis, induce the generation of new MRC units, and trigger an integrated physiological response of metabolic and functional remodeling. Several molecules are involved in the detection of specific signals of mitochondrial activity, such as NAD^+:NADH, AMP:ATP ratios, or acetyl-CoA levels. That is why alterations in this fragile equilibrium conduct to a dysregulated mitochondrial activity, leading to the altered pathophysiological response observed in, among others, MetS.

Similarly, mitochondrial activities depend on the abundance of these organelles, their morphology, and their functional properties, regulated, among others, by mitochondrial dynamics [46–48]. Mitochondria are permanently fusing and fissioning with each other, allowing the renewal of genetic, structural, and functional components. It is especially the mitochondrial fusion process, which has been reported to allow the exchange of mitochondrial content, including mitochondrial DNA (mtDNA), which may buffer partial defects and transient stresses [49,50]. The loss of fusion and fission abilities results in altered mitochondrial populations, which leads to the most common NCDs [51].

To summarize, mitochondrial dysfunction can be due to several levels of deficiency and lead to different pathological features. All their levels of function are closely related, affecting one each other, rarely having isolated effects. In the case of MetS, reduced biogenesis conducing to variations in mitochondrial number, altered membrane potential, leading to defective energy production and defects in the activity of oxidative proteins, has been reported, all together linked with the accumulation of ROS in cells and tissues [52].

4.2. Some Interactions with Other Cell Structures

Mitochondria are not isolated organelles inside the cell, so interactions with other structures must be considered in health and disease. For instance, interactions with lysosomes [53] allow the degradation and turnover of defective or unnecessary mitochondrial by mitophagy, important to maintain mitochondrial quality control (MQC). The failure of the MQC system has been described in NCDs patients [54–56], demonstrating the importance of mitochondrial interactions with other eukaryotic cell structures.

Another important interaction of mitochondria to support cell functionality is with the endoplasmic reticulum (ER) [57], also known as mitochondria-associated membranes, MAMs, which allows another level of organelle and cell regulation. Alterations in MAMs have been demonstrated in NCDs patients [58–60] due, in part, to increased ER stress but also to mitochondrial dysfunction.

4.3. Some Mitoregulators

4.3.1. Epigenetics and Sirtuins

Epigenetic modifications in DNA, which include acetylation, methylation, and ubiquitination, among others [61], determine the transcriptional machinery accessibility to the genome [62]. However, these regulatory modifications are not limited to DNA and located in the nucleus, they can also target RNA, proteins, and mitochondria, and be associated with disease [63,64].

One well-known epigenome effector, which work as cellular sensors of metabolic status, are the NAD^+ dependent histone deacetylases sirtuins [65]. Sirtuins are a family of seven proteins involved in different biological functions [66] such as DNA repair [67], apoptosis control [68,69], ROS and oxidative stress regulation [70], together with their modulatory activity over enzymes from catabolism to anabolism [71]. Some of them have mitochondrial location or activity [66,70].

The most studied sirtuins are Sirt1 and Sirt3. Genetic or pharmacological Sirt1 inhibition can induce insulin resistance (IR) [72], and genetic Sirt1 variations have been associated with human energy expenditure and obesity [73–75]. Moreover, the fasting state increases NAD^+ levels and activate metabolism through Sirt3 [76]. In the absence of Sirt3, cell and mitochondrial proteins become hyperacetylated, contributing to the progression of metabolic diseases such as MetS [77,78].

4.3.2. miRNAs, mitomiRs, mitoRNAs, and xenomiRs

Non-coding ribonucleic acids (ncRNAs) are a wide heterogenous group of regulatory molecules described in the last decades. Inside this group, there are micro-ribonucleic acids (miRNAS), short molecules that prevent mRNA translation or induce transcripts degradation, thus preventing the generation of the protein and associated function. These molecules are mostly located in the cytoplasm, but there is a subset of about 150 different miRNAs, named mitomiRs, that have been also found in the mitochondrial fraction [79]. Furthermore, the transcriptome of mitochondrial genome has also revealed that it encodes multiple non-coding RNAs, which are named mitoRNAs [80]. Furthermore, in the past few years, these molecules have been associated with several NCDs [81–83], including MetS [84]. Interestingly, dietary changes in MetS patients associated with clinical improvements have been linked to changes in miRNAs profile [85], as later depicted.

Nutrients can interplay at different levels with the human body. Their components can be the anabolic precursors for the synthesis of complex molecules needed for cell survival. They can also interplay at the chemical level with cell constituents; this is the case of the antioxidant effect. The last findings support the idea that they can also regulate the expression of our genes. This is the case with the xenomiRs, or food-derived miRNAs, exogenous miRs that emulate the function of our endogenous miRs, exerting an additional mechanism of regulation for the expression of our genes. Despite there being only few data available and plenty of polemics, some studies revealed an important concentration of rice [86] or milk [87] xenomiRs in the plasma, after a dietary intervention. On the contrary, other publications were unable to detect them [88,89]. Additionally, in case these xenomiRs were absorbed, there is the crucial need to understand if they exert any function in our cells, as endogenous miRs do. What is certain is that this field of study will be of growing interest in the next coming years.

4.4. Contribution of Mitochondrial (Dys)Function to MetS

The association between MetS and mitochondrial (dys)function has been suspected since long ago and its contribution to the deterioration of the metabolic risk factors involved in the disease has been studied.

Westernized diets, that usually include high caloric intake (high fat and/or sugars) might promote an increase in the MRC activity that could lead to the overproduction of mitochondrial ROS [90]. In turn, the oxidative stress leads to reduced ATP production and decrease of the overall metabolism in the cell, which is tightly associated with MetS [91–93]. Moreover, oxidative stress might contribute to the appearance of insulin resistance (IR), defined as a decreased cellular response to physiological insulin levels [94]. However, it remains unclear whether mitochondrial (dys)function is the main cause of IR or vice-versa [95,96].

Furthermore, oxidative stress directly interferes in lipid oxidation, which results in an increased cellular lipid accumulation (mainly diacylglycerols and ceramides), a situation that inhibits insulin signaling [94,97,98]. This situation affects the translocation of the glucose transporter type 4 (Glut-44) in insulin-sensitive tissues such muscle or fat [99], which leads to circulating glucose accumulation and consequently, to hyperglycemia [100].

Moreover, lipids and free fatty acids (FFA) also accumulate in the circulatory system, leading to hypertriglyceridemia [101], which in turn promotes the increase of visceral adiposity and a modification of fat distribution, and consequently the increase of waist circumference. This situation is caused by the inability of adipose tissue to store the energy excess, so the FFA overload is accumulated in the muscle, heart, liver, or as subcutaneous fat [102]. Under these conditions, fat redistribution is accompanied by macrophages infiltration, which secrete proinflammatory molecules such as interleukin-6 (IL-6), tumor necrosis factor (TNF), or C reactive protein (CRP), generating a low-grade chronic inflammation state in MetS patients [103,104]. Furthermore, abdominal obesity correlates with systemic levels of oxidative stress biomarkers [105,106] and defective mitochondrial biogenesis, manifested by impaired mitochondrial dysfunction, which includes alterations in oxidative metabolism, low mitochondrial gene expression, and reduced ATP generation [107,108]. Similarly, mitochondrial alterations, increased oxidative stress, and low-grade chronic inflammation contribute to the etiology of hypertension [109,110]. Finally, oxidative stress in also one of the main triggers of the formation and progression of the atheroma plaque responsible of cardiovascular events included in the MetS.

5. Nutrients (Well-Being Through Feeding)

One of the best options to avoid, delay, or minimize risk factors contributing to MetS is changing lifestyle routines, including a balanced diet.

5.1. Monounsaturated Fatty Acids (MUFAs)

MUFAs are fats with one unsaturation in their carbon chain. Dietary MUFAs can have different origin, thus in Western diets, the supply is through foods of animal origin, whilst the main MUFAs source in south European countries is extra virgin olive oil containing oleic acid [111]. Other sources of MUFAs are the rest of vegetables oils and nuts such as macadamia nuts, hazelnuts, or pecans [112].

5.1.1. Some Extra Virgin Olive Oil Everywhere. Oleic Acid

Some cellular model studies have reported that oleic acid can have a positive role in mitochondrial function. In fact, one study from in vitro modeling of β-pancreatic cells showed that oleic acid enhances antioxidative defense [113]. Moreover, alternative studies demonstrated that oleic acid supplementation reduces ROS generation and protects mitochondria from oxidative stress or apoptosis [114,115]. Furthermore, oleic acid has been also reported as an anti-inflammatory modulator through AMP-activated protein kinase (AMPK) activation and its activity among different targets like nuclear factor kB (NF-kB) inhibition, and consequently, decreasing cytokines secretion such as TNF [116–118].

In turn, oleic acid also enhances fatty acid β-oxidation through AMPK response and interaction with Sirt1 and the peroxisome proliferator-activated receptor gamma coactivator 1-alpha PGC-1α [119,120], the master gene regulator of mitochondrial remodeling and biogenesis. Finally, oleic acid has been demonstrated to increase the carnitine palmitoyl transferase 1 (CPT-1) level promoting the fatty acid transport into mitochondria for β-oxidation [121,122]. Of note, most of these mechanisms have been described using in vitro models responding to higher concentrations of oleic acid compared to those obtained by food intake. Interestingly, several clinical benefits have been described with lower doses (see Section 5.1.2). Further and deeper investigation is needed to validate the mechanisms and dose-response effects of oleic acid, often observed in proof-of-concept studies.

5.1.2. Extra Virgin Olive Oil Against MetS

Systematic review and meta-analysis of cohort studies have demonstrated the positive effects of oleic acid decreasing mortality risk (11%) and cardiovascular risk (12%) [123,124]. Focusing attention on MetS, dietary MUFAs showed similar results [112]. In fact, clinical trials have demonstrated a reduction of MetS risk factors such as blood pressure or total low-density lipoprotein (LDL) cholesterol [125]. Moreover, also an improvement on insulin sensitivity and inflammatory response

have been demonstrated using oleic acid in cell models [126] and randomized clinical trials [127]. Finally, recent systematic review on human intervention studies with enriched oleic acid diets demonstrated that this intervention can reduce central obesity and abdominal fat [128].

5.2. Polyunsaturated Fatty Acids (PUFAs)

PUFAs are fats with more than two unsaturations in their carbon chain that can be divided into two main groups. Omega-3 fatty acids (α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid) mainly found in blue fish, eggs, and walnuts. In turn, the omega-6 group (linoleic acid, arachidonic acid, and docosapentaenoic acid) are mostly founds in vegetable oils, such as sunflower, soybean, corn, and canola oils.

5.2.1. More Blue Fish and Less Vegetal Oils. Omega-3 vs. Omega 6

Omega-3 PUFAs have been reported to act as mitochondrial protectors, as they reduce ROS production and inflammation through the activation of AMPK. In turn, AMPK activates PGC-1α which, together with the decrease in fission genes such as dynamin related protein 1 (DRP1), stimulates mitochondrial function and fusion processes [129]. PGC-1α triggers different signaling pathways that promote mitochondrial biogenesis, β-oxidation, glucose utilization, antioxidants detoxification, and the activation of uncoupling proteins, which leads to a decrease in lipid accumulation and a reduction of ROS [130–133]. It has also been described to attenuate ER-stress and the subsequent disruption of Ca^{2+} homoeostasis. All these actions decrease the expression of pro-inflammatory cytokines and attenuate the inflammasome, which improves insulin sensitivity [134]. Some animal studies and clinical trials demonstrate that these actions would be triggered by changes in miRNAs profiles, among others [135,136]. In addition, the reduction of the omega-6/omega-3 balance and the omega-3 incorporation into mitochondrial membranes increase its viscosity. This situation improves mitochondrial function and avoids cell dysfunction and cell death [137]. However, the disbalanced omega-3/omega-6 ratio in detriment of omega-6 may also lead to adverse consequences, since excessive omega-3 PUFAs may eventually trigger pro-oxidant and pro-inflammatory environments [138]. Thus, the conservation of the omega-3/omega-6 ratio balance is key to avoid detrimental effects (Figure 3).

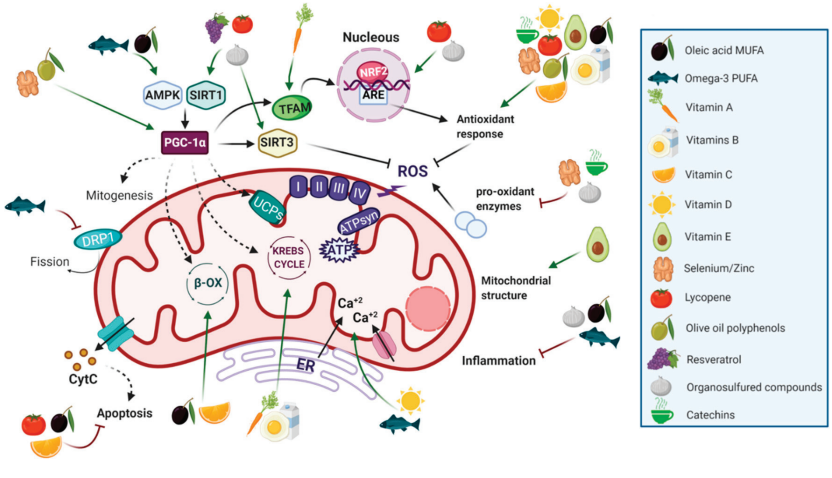


Figure 3. Principal nutrients with beneficial effects on mitochondrial function. Oleic acid, omega-3, selenium and zinc, resveratrol, lycopene, and the organosulfur compounds (OSCs) activate numerous pathways, among them, direct or indirectly, PGC-1α signaling, one of the main coactivators and master gene regulator of energy metabolism and mitochondrial biogenesis. The activation of the PGC-1α promotes mitogenesis, β-oxidation, glucose utilization, uncoupling, and antioxidant detoxification.

Moreover, vitamin A and vitamins from the B group are essential for a correct functioning of the glycolytic energy system, and vitamin C and oleic acid for the functioning of β-oxidation, among others. One of the main effects of oleic acid, lycopene, organosulfur compounds (OSCs), vitamins B, C, D, selenium, and zinc, catechins and olive oil polyphenols (OOPs) in our cells is the decrease of oxidative stress by both promoting an antioxidant response and inhibiting pro-oxidant enzymes. Vitamin E also acts as an antioxidant and maintains a correct mitochondrial structure, while omega-3 inhibits mitochondrial fission. Omega-3 and vitamin D are involved in calcium homeostasis, among others. Vitamin C, lycopene, and oleic acid have been reported to inhibit apoptosis, while oleic acid, omega-3, and OSCs reduce inflammation.

5.2.2. Omega-3 PUFAs Against MetS

It is still controversial whether omega-6 PUFAs exert pro-inflammatory or anti-inflammatory effects by themselves [139]. However, it is known that an unbalanced omega-6/omega-3 ratio in favor of omega-6 PUFAs is highly prothrombotic and proinflammatory. The high amounts of omega-6 PUFAs present in Western diets, together with the very low amounts of omega-3 PUFAs, leads to an unhealthy omega-6/omega-3 ratio that can reach a 20:1 proportion (instead the expected 1:1) [140], contributing to atherosclerosis, obesity, and T2D [141] (Figure 4).

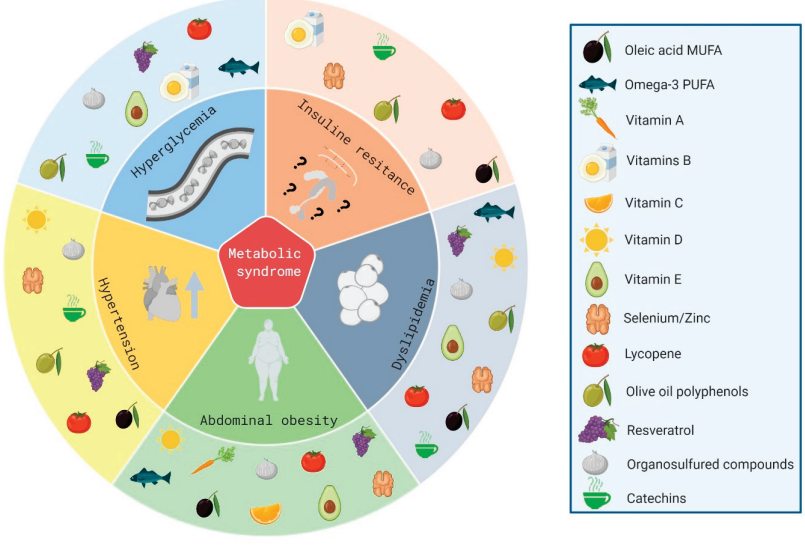


Figure 4. Main nutrient impact on metabolic syndrome (MetS) components. The MetS is a constellation of pathologic conditions which includes hyperglycemia, insulin resistance, dyslipidemia (hypertriglyceridemia and low high density lipoprotein (HDL)-cholesterol levels), central obesity, and hypertension. Several studies have demonstrated that nutrients such as oleic acid monounsaturated fatty acid (MUFA), omega-3 polyunsaturated fatty acids (PUFAs), vitamins A, B, C, D, and E, selenium and zinc elements, lycopene, olive oil polyphenols, resveratrol, organosulfured compounds, and catechins have a positive impact on MEtS components, improving the onset and development of the disease.

Moreover, animal studies have shown that omega-3 improves insulin sensitivity and reduces visceral fat storage [142,143]. Different randomized clinical trials have proven that omega-3 supplementation produces favorable hypolipidemic effects, a reduction in pro-inflammatory cytokine levels and improvement in glycaemia [144]. Animal and patient-based studies have shown that high omega-3 intake and low omega-6/omega-3 ratio are associated with lower MetS risk [145,146].

5.3. Vitamins

Vitamins are essential micronutrients for the proper functioning of metabolism. However, vitamins cannot be synthesized by the organism, or not in enough amount, so they must be obtained through diet. Vitamins can be classified into hydrosoluble (vitamins B and C) and liposoluble (vitamins A, D, E, and K). Their biological properties will be discussed below.

5.3.1. A Varied Diet. Vitamins B

Vitamins B comprise a set of molecules found in a wide variety of food groups such as dairy products (B2, B3, B5), eggs (B1, B3, B7, B12), fish and meat (B1, B3, B5,B6, B12), or plant-based foods (B1, B5, B6, B9). Vitamins B are essential in the KC, so deficiencies in some of these vitamins will lead to impaired mitochondrial metabolism, increased levels of mitochondrial ROS production, and decreased overall energy production [147].

Vitamin B1 (thiamin) is a cofactor of several enzymes such as the cytosolic pyruvate dehydrogenase or the mitochondrial ketoglutarate dehydrogenase; in vitro models demonstrate that its deficiency is linked to increased ROS formation [148]. Vitamin B2 (riboflavin) is essential as a prosthetic group for several enzymes catalyzing redox reactions; different studies demonstrated that its deficiency is associated with a loss of mitochondrial complex IV and induced oxidative stress [149]. Vitamin B3 (niacin) is an important cofactor involved in mitochondrial respiration reactions, glycolysis, or lipid oxidation; B3 have also demonstrated to have an important antioxidant activity in different investigations [150]. Vitamin B5 (pantothenic acid) is an important prosthetic group (CoA) involved in KC and lipid metabolism; cellular models also demonstrated an important role in the enzymatic antioxidant defense system promoting CAT, GR, or GPx [151]. Vitamin B6 (pyridoxal) have several functions in different metabolic pathways such one-carbon reactions, gluconeogenesis, or lipid metabolism, among others; even have an important antioxidant role through its activity in glutathione pathways, demonstrated in cellular models [152]. Vitamin B7 (biotin) is an important cofactor for some carboxylase enzymes involved in lipid metabolism; decreased B7 levels have been linked to increased ROS formation and impaired mitochondrial respiration [153]. Vitamin B9 (folic acid) exerts different biological functions like nucleotide synthesis or mitochondrial tRNA modification, among others; folate contributes to cellular redox states preventing oxidative stress indirectly [154]. Finally, vitamin B12 (cobalamin) is essential for nucleotide synthesis or succinyl-CoA generation; B12 deficiency has been related to indirect increased ROS levels due to glutathione dysregulation in patients [155].

5.3.2. Orange Juice. Vitamin C

Vitamin C, or ascorbic acid, is principally found in pepper (and other vegetables) and fruits (mainly in kiwi, guava, and citrus). This molecule is a potent antioxidant that activates Sirt1, triggering a signaling cascade that decrease ROS and apoptosis and also acts as a ROS quencher. Moreover, it is also involved in carnitine biosynthesis, the key factor in β-oxidation. Thus, vitamin C deficiency will lead to impaired ATP production, β-oxidation deficiency, and ROS formation [156,157], and therefore, mitochondrial dysfunction.

5.3.3. Carrot Cream. Vitamin A

Vitamin A or retinol is mainly found in carrots, sweet potatoes, or spinaches, as well as pork and beef liver, which concentrate the highest amounts. This vitamin affects mitochondrial function as it enhances the levels of the mitochondrial transcription factor A (mtTFA), a key transcription factor for mitochondrial function, and plays a key role in glycolytic energy generation, as an essential cofactor for protein kinase C delta (PKCδ), which signals the pyruvate dehydrogenase complex for an enhanced flux of pyruvate into the KC [158]. Thus, deficiency of vitamin A has been associated with decreased respiration and ATP synthesis [158–160].

5.3.4. Slight Daily Sunbathe. Vitamin D

Vitamin D is naturally present in blue fish and egg yolks, among other foods. Furthermore, vitamin D can be synthesized by the human body when ultraviolet rays from sunlight strike the skin. However, after its obtention, either from sun exposure or food, it needs two hydroxylations to be biologically active.

Many biological functions of vitamin D are mediated by the control of the vitamin D receptor (VDR) on nuclear transcription. The VDR main function is to regulate Ca^{2+} homeostasis through the upregulation of calcium transporters. Moreover, VDR protects from an excessive respiratory activity and limits ROS production by controlling mitochondrial and nuclear transcription of the proteins involved in MRC and ATP synthesis [161,162].

5.3.5. Avocado Is the Answer. Vitamin E

Vitamin E is mainly found in avocado, leafy vegetables such as spinach and broccoli, sunflower seeds, nuts, and olive oil. Vitamin E is the most important antioxidant in cell membranes. It protects mitochondrial structure and function by maintaining mitochondrial membrane integrity, allowing the recovery of respiratory function and reducing lipid and protein oxidation [163]. This protective function would be, in part, explained by the inhibition of lipid peroxidation and the increase in CAT, SOD, GR, and GPx activity demonstrated in T2D rats [164].

5.3.6. Vitamins Against MetS

The role of vitamins in mitochondrial metabolism and their antioxidant properties suggest that they might play a role in the prevention of MetS. Indeed, a case control-study showed that plasma levels of vitamins A, C, E, and D were significantly lower in MetS patients compared to healthy subjects [165,166].

Different animal studies have suggested that vitamin A supplementation ameliorates obesity through UPC1-mediated thermogenesis and delays the appearance of T2D by increasing mtTFA [158,167]. Vitamin B7, biotin deficiency has been linked to impaired glucose tolerance, hyperglycemia, and decreased glucose oxidation in animal studies [168] and a cross-sectional study showed that high levels of vitamin B12 are protective to MetS [169]. Regarding vitamin C, different patient-based studies have suggested that intake or supplementation decreases the risk of MetS and improves the quality of life of MetS patients [169,170], especially when combined with physical exercise [171,172]. Moreover, a cross-sectional study with more than 2000 patients demonstrated that deficiency of vitamin C was associated with an increased likelihood of MetS [169]. Several epidemiologic studies have established a strong association between vitamin D deficiency and hypertension, obesity, and dyslipidemia [173,174]. In a randomized clinical trial with MetS patients, vitamin E was seen to exert beneficial effects on cytokines and the lipid profile [175]. Moreover, different studies have found that vitamin E could ameliorate the pathologic conditions associated with MetS such as hyperglycemia or obesity, although more studies in patients are needed to prove the consistency of these findings [175,176].

Nevertheless, when it comes to vitamins, overconsumption may be just as bad as scarcity. For instance, an excess of vitamin A intake (with a tolerable upper intake level for adults of 3000 μg/day) [177] promotes oxidative stress and mitochondrial death [178] and it is associated with an increased likelihood of MetS [160]. A high intake of vitamin B3 (niacin), can result in niacin-induced IR [179], and when Vitamin C and E are consumed in excess, they promote prooxidants activity instead of antioxidant function [147].

5.4. Trace Elements

Trace elements are minerals present in small amounts in living tissues. Some of them are nutritionally essential and so need to be incorporated through diet, such as selenium or zinc, and others are considered nonessential (or there is not consistent evidence to regard them as essentials) [180].

5.4.1. A Handful of Nuts. Selenium and Zinc

Selenium and zinc are mainly present in nuts, mushrooms, fish, selfish, and meat, with antioxidant and mitochondriogenic properties. Selenium promotes mitochondrial biogenesis through the stimulation of PGC-1a, while the nuclear redox factor1 (NRF-1) generation exerts its antioxidant role activating both GPx and GR enzymes. Some of these positive effects would be modulated by selenium impact on miRNAs profile preventing oxidative stress and inflammation [181]. On the other hand, zinc exerts its antioxidant power by promoting SOD and CAT activation, inhibiting important pro-oxidant enzymes such as NADPH oxidase and competing with redox-active transition metals such as iron and copper for certain binding sites (cell membranes, proteins), thereby prohibiting them from catalyzing the formation of ROS and the initiation of lipid peroxidation [182].

5.4.2. Trace Elements Against MetS

Some studies have shown that adequate intake of these trace elements is associated with an attenuation of MetS risk factors. In vivo and patient-based studies have found that recommended doses of selenium (1–2 μg/kg/day) act as an insulin-mimetic, attenuating diabetes [183–185] and have cardioprotective effects (increase plasma antioxidant capacity and decrease lipid peroxidation and LDL-cholesterol) [186–188]. Additionally, a cross-sectional study including more than 2000 patients showed that dietary selenium intake was negatively associated with MetS [170]. Regarding zinc, it is involved in different protective mechanisms against obesity, IR, hypertension, and dyslipidemia, which suggests potential roles of zinc in both the prevention and treatment of MetS. However, the current scientific evidence in humans regarding the associations between zinc status and occurrence of MetS is still inconsistent [189].

Despite these healthful and wellness properties of minerals, as it happens with vitamins, excess of these elements is just as bad as a deficiency. Fortunately, severe impact on human health of vitamins and minerals is only reached at very high concentrations. For instance, doses of selenium 100 times higher than expected had adverse effects than at recommended doses by increasing the risk of T2D and promoting CVD [190–192].

5.5. Polyphenols

Polyphenols are bioactive compounds with antioxidant properties mostly found in plant-based foods [193]. According to their molecular structure, polyphenols can be categorized into two main groups: flavonoids (such as catechins) and non-flavonoids, including phenolic acids (such as ellagic acid), stilbenes (such as resveratrol), lignans (such as pinoresinol), and others (in which we find oleuropein and hydroxytyrosol). Many of them show mitochondrial effects and have been reported to modulate MetS [194].

5.5.1. A Cup of Green Tea. Catechins

Inside the flavonoids family, we find the catechins molecules, present mainly in green tea (with a total minimum content of catechins of 8%), but also in a wide variety of fruits (Table 2). It has been demonstrated that catechins can directly work as ROS scavengers and metal ions chelators and activate some antioxidant enzymes such as CAT and SOD. Moreover, they can also inhibit some pro-oxidant enzymes like NADPH-oxidase and suppress stress-related signaling pathways (as those mediated by TNF or the activator protein 1). Some in vitro studies also demonstrated an anti-inflammatory effect of catechins through changing miRNAs profile [195]. All together, these effects contribute to decrease oxidative stress and improve mitochondrial function [196].

Table 2. Summary of the principal nutrients with beneficial mitochondrial effects influencing metabolic syndrome.

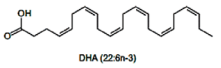
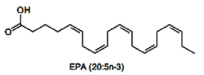
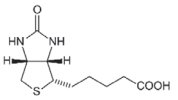
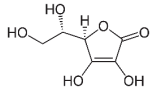
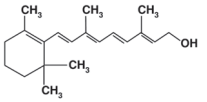
Nutrient	Molecular Group and Structure	Main Food Sources	RDA [197]	Principal Role in Mitochondria	Main Effect against Metabolic Syndrome	Main LoE	Reported References
Oleic acid	 MUFA	Olive oil Vegetables oils Nuts	20 g of olive oil/day [1]	Antioxidant Antiapoptotic Enhances β-oxidation	↓ Blood pressure	H	[125]
					Improves lipid profile	H	[125]
					↑ insulin sensitivity	H	[126,127]
					↓ inflammation	H	[126,127]
					Reduces central adiposity	H	[128]
Omega-3	 DHA (22:6n-3)  EPA (20:5n-3) PUFA	Fish and seafood Nuts and seeds	1.1–1.6 g/day (ALA)	Uncoupling β-oxidation mitochondrial biogenesis	hypolipidemic effects	H	[140,143, 144]
					↑ insulin sensitivity	H	[142,144]
					↓ inflammation	H	[140,144]
					↓ MetS risk	H	[145,146]
Vitamins B	 Water-soluble vitamin [2]	Dairy products (B2, B3, B5) Eggs (B1, B3, B7, B12) Fish and meat (B1, B3, B5, B12) Plant-based foods (B1, B5, B9)	B1: 1.1–1.2 mg/day B2: 0.9–1.1 mg/day B3: 14–16 mg/day B5: 5 mg/day B6: 1.3 mg/day B7: 0.03 mg/day B9: 0.3–0.4 mg/day B12: 0.0024 mg/day	Essential in Krebs Cycle Antioxidant	Low levels are associated with hyperglycemia and insulin resistance	H	[167,179]
					Protective to MetS	H	[168]
Vitamin C	 Water-soluble vitamin	Guava Citrus fruit Kiwi Strawberries Peppers	75–90 mg/day	Antioxidant Antiapoptotic Involved in β-oxidation	↑ the effects of physical activity in the prevention of MetS and the quality of life of MetS patients	H	[168–171]
Vitamin A	 Fat-soluble vitamin	Carrots Pork/beef liver Foie Spinach Sweet potato	0.7–0.9 mg/day	Key role in mitochondrial respiration	Ameliorates obesity	A	[166]
					Delays the appearance of diabetes	A	[158]

Table 2. *Cont.*

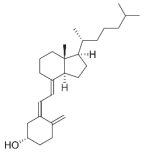
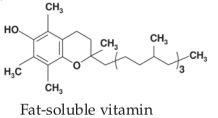
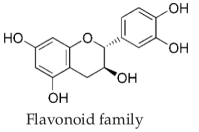
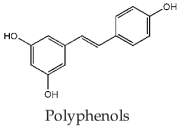
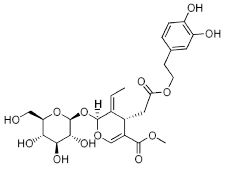
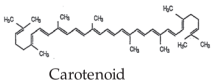
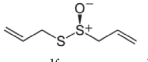
Nutrient	Molecular Group and Structure	Main Food Sources	RDA [197]	Principal Role in Mitochondria	Main Effect against Metabolic Syndrome	Main LoE	Reported References
Vitamin D	 Fat-soluble vitamin [3]	Blue fish Egg yolk Synthetized in the presence of sunlight exposure	0.015 mg/day	Controls the respiratory activity and limits ROS production	Low levels are associated with hypertension, obesity and dyslipidemia	H	[161,162]
Vitamin E	 Fat-soluble vitamin	Sunflower seeds Leafy vegetables Nuts Olive oil	15 mg/day	Protects mitochondrial structure and function (antioxidant)	↓ inflammation	H	[174]
					Improves the lipid profile	H	[174]
Selenium and Zinc [4]	NA Trace element	Nuts Fish and selfish Meat	Se: 0.055 mg/day Zn: 8–11 mg/day	Antioxidant Mitochondrial biogenesis	Insulin-mimetic	H	[183–185, 189]
					Improves lipid profile	A	[189,198]
					Cardioprotective	A	[186–188]
Catechins	 Flavonoid family	Green tea Broad beans Blueberries Black grapes Strawberries Apricots	Up to 704 mg/day [1]	Antioxidants	↓ Blood pressure	H	[199–201]
					Improve lipid profile	H	[200,202]
					Insulin-like/-enhancing activities	H	[203,204]
Resveratrol	 Polyphenols	Grapes Apples Blueberries Pistachios Peanuts	Up to 4 mg/day [1]	Mitochondrial protective agent (antioxidant)	↓ body weight	H	[205,206]
					↓ waist circumference	H	[205,206]
					Improve lipid profile	H	[205]
					↓ glucose levels	H	[205]
					Cardioprotective	H	[204,207, 208]

Table 2. *Cont.*

Nutrient	Molecular Group and Structure	Main Food Sources	RDA [197]	Principal Role in Mitochondria	Main Effect against Metabolic Syndrome	Main LoE	Reported References
Oleuropein (OL), Hydroxytyrosol (HT), Pinoresinol	 Oleuropein structure	Olive oil	20 g of olive oil/day [1]	Mitochondrial protective agent (antioxidant)	Cardioprotective	H	[204]
					↓ Insulin resistance	H	[204]
					↓ Blood pressure	H	[209]
Lycopene	 Carotenoid	Tomato Watermelon Papaya Apricots Red grapefruit Carrots Pumpkin Sweet potato	5.7–15 mg/day [1]	Antioxidant	↓ Blood pressure	H	[210,211]
					Anti-obesogenic	A	[212,213]
					improves the lipid profile	A	[214,215]
					↑ insulin sensitivity and ↓ plasma glucose	H	[216,217]
					↓ Risk of death in MetS patients	H	[211]
Allicin	 Organosulfur compounds	Garlic	1–2 g of raw garlic [1]	Antioxidant Uncoupling	Improves obesity	H	[218–222]
					Improves lipid profile	H	[223]
					↓Hyperglycemia	H	[222]
					Cardioprotective	H	[224–226]
					↓Hypertension	H	[223,227]

[1] Suggested recommended intake [228–232]. [2] Molecular structure from biotin (B7). [3] Molecular structure from cholecalciferol (D3). [4] The current scientific evidence in humans shows inconsistent results regarding the beneficial effects of zinc in MetS risk factors. RDA = Recommended dietary allowance; A = Animal studies; H = Human studies; NA = Not applicable.

5.5.2. Some Black Grapes. Resveratrol

Resveratrol is another antioxidant, mainly found in black grapes. Resveratrol is a mitochondrial protective agent. It activates Sirt1 which triggers different signaling pathways that promote antioxidant and anti-inflammatory effects, and parallelly activate PGC-1α, the master regulator of mitochondrial biogenesis. Sirt1 activation enhances β-oxidation and glucose utilization, promotes mitochondrial biogenesis, antioxidant detoxification, and the expression of uncoupling proteins [233,234]. Moreover, some of the anti-inflammatory effects of resveratrol are triggered by change in miRNAs profiles, as demonstrated in cell models [235] and clinical human trials [236]. Of note, resveratrol is one of those nutrients were herein reported beneficial in vitro effects have been observed with concentrations usually beyond those reached by food intake. Notwithstanding, and regardless the mechanism, clinically beneficial effects have also been reported [236]. Further studies are required to deepen in dose-response effects of resveratrol, their interaction with other nutrients, and host metabolism.

5.5.3. A Spoon of Olive Oil. Oleuropein, Hydroxytyrosol, and Pinoresinol

Olive oil have a great content of polyphenols such as oleuropein (OL), hydroxytyrosol (HT), and pinoresinol. Olive oil polyphenols (OOPs) are mitochondrial-protective agents for its antioxidant properties. It has lately emerged that OOPs, particularly HT, are able to induce Sirt1 expression. Moreover, Sirt1 interaction with nuclear redox factor 2 (NRF2) signaling, responsible for the transcriptional activation of antistress target genes, exerts a protective effect against oxidative stress. Furthermore, OL and HT can also scavenge ROS and OOPS have been demonstrated to activate antioxidant enzymes such as GPx and CAT. Finally, HT has also been reported to stabilize some mitochondrial complex-subunits and enhance the expression of PGC-1α and CPT-1 [204], with previously reported mitochondrial activity.

5.5.4. Polyphenols Against MetS

Different studies have proven the beneficial effects of polyphenols in the prevention of MetS. Animal studies have shown that green tea catechins lowers blood pressure by reducing ROS [199] and have lipid-lowering activities [202]. Moreover, catechins and OOPs have been demonstrated to enhance glucose tolerance and decrease events related to IR in both animal and human-based studies [203,204]. In addition, cardioprotective roles have been attributed to resveratrol and OOPs thanks to their ability to counteract inflammation and reduce LDL-oxidation [204,207,208].

In humans, a meta-analysis of 20 randomized clinical trials, with a total of 1536 participants who received green tea regularly, showed a slight decrease in systolic blood pressure and a moderate reduction of LDL cholesterol [200]. Another study with 48 MetS patients demonstrated that dietary achievable doses of blueberries significantly decreased blood pressure [201]. This blood pressure-reducing effect has also been attributed to OOPs by different intervention studies [209]. Regarding resveratrol, a meta-analysis including 16 studies, 10 human-based and 6 in vivo studies, showed that resveratrol intake meaningfully reduces the body weight, waist circumference, triglycerides, and glucose level [205]. Moreover, a meta-analysis including 28 randomized clinical trials also documented an improvement in obesity measures with resveratrol supplementation [206].

5.6. A Tomato Salad. Lycopene with Oleic Acid

Lycopene is a carotenoid found mainly in tomato, but also in other fruits such as watermelon, papaya, or red grapefruits. The lycopene content increases during different stages of fruit ripening, so the higher content of lycopene is found in ripe tomatoes [237].

However, lycopene undergoes photo-oxidation and degradation with light, which produces a decrease in bioavailability. The incorporation of lycopene in an oil phase overcomes photo-oxidation. An interesting option is the association of lycopene with olive oil, which prevents lycopene degradation

and enriches its healthy properties with oleic acid, a monounsaturated fatty acid with antioxidant properties [238,239].

Lycopene is also a powerful antioxidant and anti-inflammatory molecule. It acts as both ROS and nitrogen species scavenger [240], decreases DNA damage [241–243], and modulates the production of SOD and GPx [244,245]. Moreover, it promotes the expression of constructs containing antioxidant response elements (ARE). Regarding its anti-inflammatory effects, lycopene reduces apoptosis as well as the expression of inflammatory cytokines [246]. Lycopene also exerts lipid-lowering properties through the induction of Sirt1 activity [247].

Lycopene Against MetS

Based on the potent antioxidant and lipid-lowering properties of lycopene, different studies have assessed it as a beneficial nutrient for the prevention and treatment of MetS. Animal and human-based studies have shown that lycopene reduces blood pressure [210,211], atherosclerotic burden [248] and improves blood antioxidant capacity [246,249], has an anti-obesity role [212,213], improves insulin sensitivity, reduces hyperglycemia [216,217], and improves the lipid profile [214,215]. Moreover, a retrospective study with 2500 patients with MetS showed that higher serum levels of lycopene are associated with a reduced risk of death [250].

5.7. Garlic Seasoning Is Always a Good Idea. Organosulfur Compounds (OSCs)

The OSCs comprise a group of molecules found in garlic that are accumulated as γ-glutamyl peptides. Depending on the processing status of the garlic, different OSCs can be present: alliin and sulfur amino acids from whole cloves, allicin from crushed cloves, ajoene and vinyldithiines from stir-fried garlic, and (poly)sulfides from steam-distilled garlic oil. However, the main active metabolite responsible for most of the biological activities of garlic is allicin [251].

The OSCs are potent antioxidant molecules, they decrease the activity of pro-oxidant molecules, such as NADPH oxidase, and increase the activity of antioxidant proteins, such as NRF2 [252,253]. Moreover, they also activate Sirt3, which prevents cardiac oxidative stress and mitochondrial dysfunction [224]. OSCs have also anti-inflammatory effects, as they suppress the activation of proinflammatory molecules such as NF-kB or TNF [254,255].

OSCs against MetS

In vitro and in vivo studies have highlighted the anti-adipogenic effect of OSCs through the promotion of mitochondrial uncoupling (increasing the expression of UPC-1) and the inhibition of adipocyte differentiation (through AMPK activation) [218–221], as well as their cardioprotective effects (decrease plasmatic insulin, total cholesterol, and oxidative stress levels) [224,225]. The cardioprotective effects of garlic have also been demonstrated in patient-based studies [226].

Moreover, different intervention studies have shown that garlic supplementation attenuates MetS abnormalities: reduces hypertension [222,227,256], improves lipid profile [222,223], decreases waist circumference and fasting blood glucose [222].

6. The Impact of Gut Microbiome on MetS. between Nutrients and Mitochondria

The human intestines are home to a vast number of bacteria, archaea, microbial eukaryotes, and viruses, collectively known as the gut microbiome [257]. Nutrients can modify microbiome composition (this is the case of yogurts and pickles) and, at the same time, microbiome can condition the absorption of some nutrients and, thus, our health. That is why gut microbiome can be considered another intrinsic factor contributing to MetS, and many other conditions.

Diet is accepted as the first driver of gut microbiome composition and not only the cause of preventable diseases. In 2007, it was demonstrated that a high-fat-diet affects the intestinal barrier leading the pass of microbiota products to systemic circulation. These molecules, such as lipopolysaccharide (LPS), showed deleterious effects on glycaemic function and activated the

inflammatory response [258]. Recently, LPS has been also related to plasmatic decrease in high-density lipoprotein (HDL) cholesterol and increase in triglycerides [259,260].

In fact, the gut microbiome responds to diet composition [261], including changes depending on fat composition [262,263], fiber types [264,265], and food additives [266]. Moreov73er, some dietary interventions demonstrated changes in the microbiome population that negatively correlated with obesity and hypertension [267–269]. Some others do not. This is the case of dietary interventions with polyphenols in MetS patients, that despite decreasing the expression of MetS biomarkers, yielded to null differences in gut microbiota when compared to healthy subjects [270].

Furthermore, also gut microbiome metabolic end products like the short-chain fatty acids (SCFAs) that may play a role in MetS. Some SCFAs like butyrate appear diminished in inflammatory diseases [271,272], whilst another SCFAs study with propionate supplementation improved visceral adipose tissue levels and maintained insulin sensitivity [273]. Additionally, when produced in high amounts, these molecules have been also involved in obesity, among others [274].

Finally, gut microbiome can synthesize vitamin K, necessary for normal clotting activity, and some B vitamins, contributing to more than a quarter of the daily reference intake in the case of B3, B6, B9, and B12 [275]. All these vitamins are cofactors of some metabolic routes involved in proper mitochondrial function.

Beyond food and food compounds, some other interventions have an impact on gut microbiome and, consequently, on host metabolism, such as prebiotics, probiotics, and also fecal microbiome transplantation (FMT). Prebiotics are defined as non-digestible polysaccharides that promote "the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefit to the host" [276]. In turn, probiotics are defined as "live microorganisms which when administered in adequate amounts, confer a beneficial health effect on the host" [277]. Several studies have been conducted in animal models [278–280] and humans [281–284], demonstrating benefits such as decrease in serum glucose, visceral fat area, waist circumference, or cholesterol levels. However, these results have not been always reproduced [285,286]. Finally, FMT has demonstrated that it can help reverse obesity in rodents, although there is no evidence in humans [287]. However, FMT has demonstrated to increase insulin sensitivity in MetS patients [288,289].

All these data suggest a role of gut microbiome in MetS, conditioning the interplay and effects of nutrient intake within the organism. However, this research area is quite novel and needs further assessment to understand the complex interactions between gut microbiome and its host.

7. Diet as a Therapy

7.1. Healthy Diet Base: Which Food and in Which Context

The nutrients and bioactive compounds reviewed above are just an example of the beneficial impact of some dietary patterns to avoid the onset and development of MetS and its associated complications. They exert a multitude of healthy actions, some of them through the modulation of mitochondrial functions.

For that purpose, dietary habits should focus on what and how we eat rather than on nutrients. This means predominating plant-based foods and fish, typical from the traditional MedDiet [290] rather than supplementation containing the nutrients or bioactive compounds (except for specific situations). Accordingly, a recent meta-analysis has shown that a prudent/healthy dietary pattern is a protective factor for MetS [291].

Nevertheless, multiple additional factors must be considered to achieve a health-promoting lifestyle. For instance, proper hydration it is very important. According to the European Food Safety Authority (EFSA) the adequate intake of daily total water for women and men is 2 and 2.5 L, respectively [292]. In fact, suboptimal hydration has an impact on health [293], metabolism [294] and T2D [295].

Additionally, a healthy lifestyle must minimize elements that have negative effect on health, and particularly on MetS such as tobacco [296], alcohol [297], or physical inactivity [298].

Moreover, related to the physical inactivity, some regular exercise has a positive impact on disease and on personal well-being [299]. Physical activity enhances energy consumption and reduces the risk of prevalent diseases such as T2D, CVD, and MetS [300]. Because of the increase of energy consumption, the metabolic disturbances associated with MetS such as increased blood glucose, triglycerides, or inflammation improve [300,301].

However, we must be careful with the intensity of physical exercise and avoid acute trainings, which has been related to an increase of ROS production [302]. For that purpose, the most beneficial practice will be moderate intensity training, promoting the proper balance between the ROS generation and the increase in the antioxidant defense [303].

7.2. Ketogenic Diet and Mitohormesis

Under some extreme situations, as primary mitochondrial diseases, changes in diet recommended by trained professionals may help to reconfigure mitochondrial activity and consequently, adverse clinical features [11,304].

This is the case of the ketogenic diet (KD), based on the intake of high-fat vs. low-carbohydrates content. This situation mimics starvation, forcing the body to use fat as energy source. Fatty acid oxidation results in ketogenesis [305] in the liver, with the consequent exportation of ketonic bodies throughout the rest of the body.

This diet must be prescribed and monitored by professionals to assess benefits and manage side effects [306]. Surprisingly, KD has shown anticonvulsant properties, at the clinical level, and the modulation of mitochondrial biogenesis [307] and the regulation of the levels of hormones or neurotransmitters [308], at the molecular level. These effects can be explained, at least in part, for an adaptive response called mitohormesis. Mitohormesis can be defined as the biological response where the induction of a reduced amount of mitochondrial stress leads to an increment in health and viability within a cell, tissue, or organism [309]. In this case, fat consumption would promote modest ROS increase, stimulating cell proliferation, differentiation, immunity, and adaptations that would eventually (and surprisingly) lead to enhancing resistance to oxidative stress in mitochondria [310,311].

About 25–30% of epilepsy patients worldwide show resistance to any pharmacological management (refractory epilepsy). Experiences on KD have shown a reduction in seizures greater than 50% [312,313], although the explanation for this effect remains unclear [314,315].

In inherited metabolic diseases such as Leigh syndrome, glucose transporter type 1 (Glut-1) and pyruvate dehydrogenase complex (PDH) deficiency and in a subset of patients with complex I deficiency [305,308], KD has also demonstrated to be useful. Under KD, most patients with epilepsy become seizure free and improve speech, motor, and mental development, and ability [316] despite its use is only partially able to reverse the clinical course of the neurodegenerative condition [317].

7.3. Enhancing MRC and Energy Buffering. Mitochondrial Burst

Some other mitochondrial diseases, contrarily, require the supplementation with CoQ10, riboflavin (vitamin B2), or thiamine (vitamin B1) to enhance ETC, or with creatine monohydrate, to increase energy buffering.

In some genetic diseases affecting enzymes involved in CoQ10 synthesis [318], supplementation can improve the clinical symptoms related to this deficiency [319]. Supplementation with riboflavin, a key building block in complex I and II, has particular usefulness in acyl-CoA dehydrogenase-9 (ACAD9) deficiency, resulting in increased complex I activity [320,321] and in the multiple acyl-CoA dehydrogenase deficiency caused by mutations in electron-transport flavoprotein dehydrogenase (ETFDH) [322,323]. Thiamine has been used, alone or in combination with other molecules, in the treatment of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes), improving the symptoms of myopathy and lactic acidosis [324] and Leigh syndrome [325].

Finally, creatine binding to phosphate in the mitochondria is the main source of high energy in anaerobic metabolism. High energy demanding tissues like muscle or brain present the highest creatine levels [326]. In mitochondrial myopathy, the content of phosphocreatine is decreased [327], which is why supplementation with creatine monohydrate effectively improves the motor ability of patients with mitochondrial myopathy [328].

As explained, the complexity of dietary treatments for mitochondrial diseases requires the intervention of multidisciplinary and specialized units from clinical settings where both clinicians and dieticians design the proper treatment (including dietary interventions) for each affected patient. Although some recommendations may be similar, the dietetic support for mitochondrial diseases is set apart from the management of MetS.

8. Conclusions

The evidence herein discussed highlights the association of mitochondrial (dys)function with MetS. However, whether mitochondrial defects can be understood as a cause or consequence of MetS remains to be clarified. Similarly, whether all nutrients reach mitochondria and at which food dose do exert their beneficial effects is still controversial. In this sense, in vitro studies usually work with higher concentrations that significantly differ from those that are reached in human interventions and must be considered as models to understand molecular mechanisms difficult to approach in vivo. All approaches are needed to understand the interplay between nutrients, mitochondria, and MetS, or disease in general, where the effects of nutrients will differ also depending on their dose, their acute or chronic consumption, and their interaction with other food components or host characteristics. Despite growing interests, the complexity of reality is still far beyond current knowledge and information provided in the present review challenge new researchers to develop novel approaches. Whether nutrients exert their protective effect against MetS exclusively through mitochondria or by deploying a panoply of molecular effects beyond it, is also under consideration, at least for some of them.

However, some mitochondrial effects have been extensively documented in cell and animal models, as well as in patients. In particular, the above reviewed nutrients and bioactive compounds have been proved to improve mitochondrial function. In fact, oleic acid, omega-3, vitamins B and C, selenium/zinc, polyphenols, lycopene, and OSCs have been documented to improve mitochondrial antioxidant defense and reduce oxidative stress; oleic acid, lycopene, and OSCs have a potent anti-inflammatory role; vitamins A, B, and C are essential for mitochondrial energy generation; vitamin D and omega-3 regulate calcium homeostasis; vitamin E protects the mitochondrial structure; and selenium/zinc and some polyphenols stimulate mitochondrial biogenesis. At the same time, the consumption of all these elements has been associated with the reduction of MetS risk factors.

All this evidence confirms that a balanced diet represents a valuable therapeutic strategy to improve the global metabolic state, among others, through the enhancement of mitochondrial function. However, is not the intake of any of these isolated compounds by their own, as dietary supplements, but the synergic interplay of them all in foods and meals which makes the difference (for instance, lycopene of tomato with olive oil). Similarly, the excess of most of them in a diet is as bad as their absence. This is the case of the antioxidant paradox, since most of antioxidants, upon athreshold, become prooxidants compounds. In most cases, the balance is the key to obtain healthy effects within a diet. This is also the case of the intake of some foods such as fatty fish, with healthy effects for their composition in omega 3, but also prone to accumulate traces of some heavy metals and other pollutants with toxic effects at the nervous but also the cardiovascular system [329,330]. In these cases, moderate consumption is the best choice to promote healthy benefits.

Additionally, diet must not be the unique lifestyle intervention to ameliorate mitochondrial activity and MetS. In this regard, moderated physical activity has been also demonstrated to prevent and/or improve the metabolic alterations related with mitochondrial dys(function), as well as MetS, together with avoiding mitochondrial toxic drugs (as tobacco or alcohol).

In conclusion, following a balanced diet, like the MedDiet or traditional Asian diets, protects against the MetS (and many other diseases). The best medicine is in our hands; cheap, tasty, and close. However, the exact formula does not exist, maybe because it is specific for each subject. Despite all the advances in understanding the role of nutrition in mitochondrial function, the perfect dietary patterns and nutrients combination to preserve/enhance mitochondrial activity remains unclear, as well as how these dietary patterns get translated in enhanced metabolic health. For that purpose, further studies must be undertaken to fully understand the impact of a balanced diet on global/mitochondrial health. Moreover, a better comprehension of some novel intrinsic factors, such as gut microbiota interactions with nutrients and host metabolism, and extrinsic factors, such as the induced epigenetic response to dietary patterns, would contribute to the understanding of the complexity of the nutritional impact on health. Such findings should be effective for the development of the appropriate dietary interventions that may lead to avoid the onset and the development of one of the major health problems in the modern society, MetS. Interestingly, personalized medicine and individual dietary patterns will probably set the path for novel therapeutics and preventive medicine in coming years.

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nutrients

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Review

All You Can Feed: Some Comments on Production of Mouse Diets Used in Biomedical Research with Special Emphasis on Non-Alcoholic Fatty Liver Disease Research

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Abstract: The laboratory mouse is the most common used mammalian research model in biomedical research. Usually these animals are maintained in germ-free, gnotobiotic, or specific-pathogen-free facilities. In these facilities, skilled staff takes care of the animals and scientists usually don't pay much attention about the formulation and quality of diets the animals receive during normal breeding and keeping. However, mice have specific nutritional requirements that must be met to guarantee their potential to grow, reproduce and to respond to pathogens or diverse environmental stress situations evoked by handling and experimental interventions. Nowadays, mouse diets for research purposes are commercially manufactured in an industrial process, in which the safety of food products is addressed through the analysis and control of all biological and chemical materials used for the different diet formulations. Similar to human food, mouse diets must be prepared under good sanitary conditions and truthfully labeled to provide information of all ingredients. This is mandatory to guarantee reproducibility of animal studies. In this review, we summarize some information on mice research diets and general aspects of mouse nutrition including nutrient requirements of mice, leading manufacturers of diets, origin of nutrient compounds, and processing of feedstuffs for mice including dietary coloring, autoclaving and irradiation. Furthermore, we provide some critical views on the potential pitfalls that might result from faulty comparisons of grain-based diets with purified diets in the research data production resulting from confounding nutritional factors.

Keywords: animal experimentation; diet; nutrition; ingredients; lard; fibers; fructose; diet coloring; autoclaving; irradiation

1. General Aspects of Mice in Biomedical Research

The laboratory mouse derived from the house mouse (*Mus musculus*) has been first used in biomedical research as a model system since the 17th century [1]. The earliest documentation of the use of mice in scientific research was done in the year 1664 in England, where Robert Hooke in his study used this animal model to study the biological consequences of an increase in air pressure [1]. In the 19th century, mice were used for a couple of breeding experiments, in which coat color or behavioral mutations were studied. Since that, these rodents have been used in many research areas. In 1981, a first genetically engineered transgenic mouse model was introduced that expressed the herpes simplex virus thymidine kinase [2].

Thereafter, both transgenic and knockout mouse models have become essential tools in the field of immunology, oncology, toxicology, genetics, and many more. These models allow the determination of the general consequences of alterations in individual genes and their cooperation with other genes. Particularly, inbred mice, which are "isogenic organisms" (nearly) identical to each other in genotype and phenotype, are frequently used for such studies. In respective experiments, these highly similar "linear test animals" are most suitable to establish reproducible results and conclusions. Moreover, testing in mice is a central part of drug development for humans, in which they are vital as a means for pre-clinical safety and efficacy testing before starting a human trial with a candidate drug. Therefore, it is not surprising that experimental work in mice has developed as an integral part of biomedical research in the building of basic knowledge. Exemplarily, mice experiments have developed as the gold standard to confirm a proposed disease-associated mechanism in hepatology research, in particular, non-alcoholic fatty liver disease (NAFLD) [3]. Specialized protocols have been developed closely mimicking typical clinical situations, including cholestasis, poisoning, metabolic injury, portal hypertension, inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma [3]. During the last decade, the individual steps in such procedures were summarized in highly standard operating protocols (SOPs) to achieve uniformity in performance and outcome [4].

At the end of 2013, the seventh report on the statistics on the number of animals used for experimentation and other scientific purposes in the member states of the European Union (EU) was published [5]. This concise dispatch contains detailed information about the number of laboratory animals used for biomedical research in 2011 in the 27 member states with the exception of France. According to this report, a total of 11.5 million animals were used in biomedical research in 2011. From these, mice are the most commonly used species with 60.9% (~7 million animals) of the total use [5]. Although precise numbers for the worldwide total numbers of mice in biomedical research are not really available, first conservative estimates of annual laboratory animal use suggested at least 115.3 million animals to be sacrificed for scientific purposes in the year 2005 [6]. If globally the frequency of mouse usage in all kinds of animal studies is the same as in the EU (~61%), this means that about 70.2 million mice are annually sacrificed by scientists and clinical researchers. However, the strict implementation of the three Rs (replacement, reduction, and refinement) concept for animal experimentation proposed by Russell and Burch in 1959 [7] that is now mandatory standard in biomedical research in many countries [3], the introduction of relevant replacement methods [8], and finally the increasing political and public concern about animal experimentation influencing people's view toward the use of animals in research [9] have led to a significant reduction in animal number [5].

Nonetheless, all these laboratory mice bred for scientific purposes and kept in laboratory must be supplied with food. When estimating that each of the 70.2 million mice typically eat 3.5–3.75 g of food per day (10–15% of their body weight) [10] and assuming that the life span of a mouse in a typical setting of an Institute for Laboratory Animals Science is about six months (Tolba and Weiskirchen, unpublished), it can be calculated that about 44.23–47.39 million kg dietary food products are necessary to feed these mice. Surely, this value is a high underestimation because potential losses due to throwing away, passing of expiration dates, unwanted food spoilage, and many other circumstances have not been taken into account in this simplified calculation. Moreover, based on special requirements necessary in the individual mice experiments, a large variety of companies have developed dedicated to developing and providing products that meet the unique challenges for all kinds of experiments.

Such "special needs" are considered in products manufactured and marketed as "custom diets". In comparison to "standards diets" or "chows", these products are more expensive in formulation because they require costly dietary manipulation such as the addition of vitamins, minerals, special fats, cholesterol, proteins, dietary fibers, drugs or other compounds. Unfortunately, in the literature the terminology of diets is used somewhat inconsistently. Different reports use terms such as "mouse diet", "rodent chow", "custom diet", "defined diet", "purified chow", "special diet", "purified ingredient diet", "grain-based diet", "standard chow", and many others. However, there are basically only

two types of diets, namely the "grain-based diets" and "purified ingredient diets". While "grain-based diets" are made out of grain, cereal ingredients, and animal by-products, "purified ingredients diets" are composed of highly refined ingredients [11].

In addition, rodent diets may require sterilization techniques when animals sensitive to normal or opportunistic microbes, such as immune compromised or germ free mice, are investigated. Food sterilization or decontamination is possible by exposure to γ-irradiation or by high-vacuum autoclaving. Highly sensitive diets can also be vacuum-, gas-, or modified atmosphere-packed (i.e., nitrogen-purged), which minimizes the risk of spoilage by oxidation.

Therefore, all these circumstances illustrate that the production of standard and customized diets intended to be used as mice feed is a complex business and a science in itself. We here will summarize some important issues on leading manufacturer, the production and diversity of mice research diets, their ingredients, and treatments during production.

2. Producers of Mouse Diets

Usually most laboratory mice are kept in centralized, well-designed, managed animal facilities, which allow efficient, economical and safe animal experiments. Depending on the design and size of the animal facility, the mice are either kept in high barrier, specified pathogen free (SPF) areas with restricted access to animal facility staff only or in low barrier (conventional) areas with additional access for licensed scientists. Usually, trained and skilled staff takes care of the animals and scientists usually don't care about the formulation and quality of diets the animals receive during normal breeding and keeping. However, scientists investigating certain immunodeficient strains, analyzing diet-induced impairments, or conducting experiments in which nutritional factors interfere with the outcome of their experiments are more interested in the products feed to their mice.

Grain-based diets are commercially manufactured in an industrial process and the safety of products should be addressed through the analysis and control of all biological and chemical materials used in the production process. Companies with an international reputation for quality often are certified by quality assurance systems and work in strict accordance with the guidelines provided by either local (e.g., England: Food Standards Agency, FSA; France: French Agency for Food, Environmental and Occupational Health & Safety, ANSES) and/or international institutions such as the International Organization for Standardization (ISO), the European Commission (e.g., https://ec.europa.eu/food/safety/animal-feed_en) or the good manufacturing practices (GMP) of the World Health Organization (WHO). If necessary, these specifications must then be adapted locally by the responsible animal welfare authorities. These guidelines or directives ensure that manufacturing, testing processes, and labeling and batch processing record are clearly defined, validated, reviewed, and documented, providing the basis to conduct good laboratory practice (GLP) studies. Furthermore, these regulations guarantee that mouse diets are prepared under good sanitary conditions and truthfully labeled to provide information of all ingredients. As such, they are rather similar to the guideline used for human foods.

3. The Production Process

3.1. Grain-Based Diets

Grain-based diets are made in "closed formulas" containing natural ingredients such as soybean meal, ground corn, fish meal, animal byproducts, and very high levels of both soluble and insoluble fibers [12]. In addition, such chows frequently contain non-nutritive but biologically active compounds such as phytoestrogens and toxic heavy metals. Grain-based diets for biomedical research purposes are made in accredited facilities using SOPs that guide all facets of diet production. Each diet formula is manufactured by a fixed formula or are produced by supplementation of a "constant nutrition" designed and supervised by a nutritionist. Depending on the ingredients, fixed formulas can reduce variation of nutrients from batch-to batch. However, grain-based diets may still be subject to variation

due to the complex nature of the ingredients in these diets, which contain multiple nutrients and non-nutrients known to be subject to variation. Different work steps are integrated into a linear sequence within the production process (Figure 1).

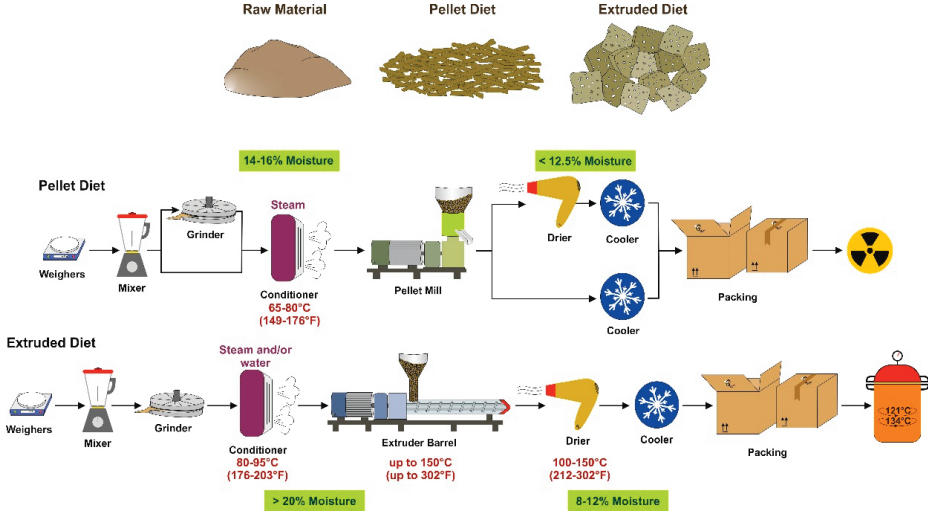

Figure 1. Schematic overview about the production process of mouse diets. Pellet and extruded diets are produced from the same raw materials. In a first step, the different ingredients are put together in the intended proportion, mixed, grinded to the desired density, and moistened to a desired moisture content (pellet diets: ~14–16%; extruded diet: >20%). Pellet diet is then processed in a pellet mill and dependent on the water content included either directly cooled or dried to a moisture content lower 12.5%. Thereafter, this diet is packed and sterilized for example by irradiation. In contrast, during the production of an extruded diet, the conditioned materials are then forced through an opening of a perforated plate or die to create a product in desired shape and size. It is then dried to moisture content of 8–12%, cooled, and packed. These diets are more or less germ-free because of the high temperature in the extruder barrel and drier. If necessary, these diets can be further sterilized by autoclaving before use. However, the high temperature during the extruding process already warrants low concentrations of microorganisms.

Key steps in the production process of grain-based diets in large quantities are the choice and delivery of raw materials (Figure 2A–C), quality control of these materials (Figure 2D–E), compilation and assignment of lot numbers (Figure 2F), and the computer-controlled mixing of individual compounds (Figure 2G–I) in suitable mixers. When producing an "extruded diet", the mixed substances are then mixed with steam and hot water and fed into the extruder barrel and forced through the die opening to form a product in desired shape and size (Figure 2J–L). During this process the quality and moisture content is continually monitored and/or adjusted. The final product is then packed in multilayer paper bags or sacks (Figure 2M) and screened for unwanted stray metal particles by passing through an in-line metal detection capability (Figure 2N). The packed diets are then transported and stocked in suitable facilities with controlled temperature and humidity conditions to avoid food spoilage (Figure 2O). From there, the diets are quickly retrievable on demand.

Figure 2. Overview of some work steps in the production of an extrusion diet in large scale. (**A**,**B**) The first step in production of mouse diets is the selection, procurement and approval of high quality bulk ingredients. In this regard, each company might have a different source of raw materials. (**C**–**E**) From each bulk, samples are taken and screened by a well-trained technician for mycotoxins (e.g., aflatoxin, vomitoxin, fumonisin), protein, fat, fiber, and moisture using near infrared spectroscopy and approved/certified test kits. (**F**) When quality is assured, each lot of raw material receives a lot number that is used to track each ingredient through the entire production process. (**G**–**I**) The different ingredients are mixed together in a certified mixer, in which flow from ingredient bins, scales and processing is critically monitored. (**J**–**L**) To produce an extruded diet form, the mixed ingredients are sent first to a post grind hammer mill and then to an extruder, in which the mixture is forced through a die. In this device, the product is expanded by a stream that is injected under pressure. In a next step, the product is passed through a dryer and several screeners to ensure that no metallic traces or other unwanted compounds are passed through. The moisture and bulk density of the product is evaluated and recorded. (**M**,**N**) Finally, the diet is packed in packing lines and once validated for unwanted stray metal particles by an in-line metal detection capability. (**O**) All diets are stocked in suitable facilities from which they are quickly retrievable on demand. All images were kindly provided by Dr. Jörg Lesting (Envigo Teklad Diets, Madison, WI, USA). A well-arranged movie showing the complete manufacturing process is viewable online at: https://www.envigo.com/p/teklad/.

3.2. Purified Diets

Purified diets are composed of refined ingredients that have undergone further processing and the composition is open to the researcher [12]. They usually contain a standardized and balanced quantity of proteins, carbohydrates, fats and fibers provided as a mixture of casein, corn starch, soybean oil and cellulose. Therefore, these diets should be always constant in composition from batch to batch. Importantly, they can be individually tailored with all kinds of compounds and further changed in regard to their relative amount of standard ingredients.

More specialized diets produced on request of the customer are usually prepared in much lower quantities. The production process is more laborious and the equipment used such as the mixers are much smaller (Figure 3A). Furthermore, most of these diets are irradiated and are delivered in smaller heat-sealed packages. To avoid mix-ups of these customized diets with other diets, these products are frequently colored with artificial food colors. Therefore, the appearance of these diets can be highly diverse (Figure 3B). Of course, such products are more expensive because of the labor-intensive work process and the often expensive ingredients requested by the customer.

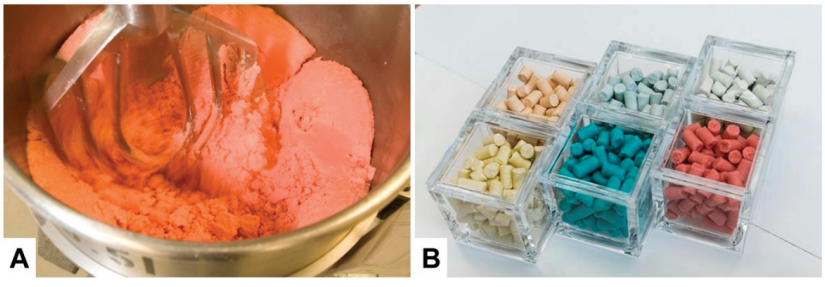

Figure 3. Production of customized purified mice research diets. (**A**) The manufacturing of a mouse diet used for basic and clinical research is a complex and highly-controlled process resembling that of producing bakery products for humans. Shown is a mixer in which the ingredients are blended. After that a known amount of water (based on the diet formula) is added and the product is properly shaped. The wet powdered diet is forced through a spinning die and without added heat, the pellets from their cylindrical shape, are cut to approximately the same length and then dried under low humidity conditions to remove the water added for pelleting. Dependent on the production process, different amount of diet ranging from several kilograms to several tons can be produced in a single batch. (**B**) Color coding by the addition of non-toxic dyes allow discriminating the different food products in animal facilities, in which animals are kept requiring different nutrients. The images depicted were kindly provided by Dr. Matthew Ricci (Research Diets, Inc., New Brunswick, NJ, USA).

High-fat diets (HFD) are widely used in studies of diet-induced obesity (DIO) and metabolic injury [13]. Diets enriched with different dietary fibers such as barley beta-glycan, apple pectin, inulin, inulin acetate ester, inulin propionate ester, inulin butyrate ester or combinations thereof have recently been shown to induce specific differences in cecal bacteria composition [14]. The beneficial effects of inulin-enriched diets and their modulatory role in microbiota composition were also sufficient to reduce inflammatory gene expression in hippocampus of APOE4 transgenic mice that develop systemic metabolic dysfunction and symptoms of Alzheimer's disease [15]. In line, the supplementation with galactooligosaccharide improved the intestinal barrier in hyperlipidemic mice lacking the low-density lipoprotein receptor (LDLR) [16]. In mice, dietary fructose impairs mitochondrial size, function, and protein acetylation, thereby decreasing fatty acid oxidation and development of metabolic dysregulation [17]. Strikingly, the exposure of mice in utero and in the pre-weaning period to a methyl donor-supplemented diet provoking DNA methylation resulted in significant attenuation of repetitive motor behavior development that persisted through early adulthood [18].

All these examples show that the diet has tremendous impact on mouse health and disease and that profound changes in the composition of diets may affect the reproducibility or outcome of mice experimentations. The knowledge of the composition of a diet during an experiment is highly crucial to maximize experimental reproducibility requiring highly standardized conditions.

To reduce experimental variation among laboratories, the American Institute of Nutrition (AIN) established a committee in 1973 with the aim to establish general guidelines in preparing dietary standards for nutritional studies with laboratory rodents [19]. These recommendations should help scientists with limited experience in experimental nutrition and facilitate interpretation of results among experiments and laboratories [19,20]. The first diets introduced by the respective committee, i.e., AIN-76 and AIN-93, contained fixed formula supporting growth, reproduction and lactation [20]. Although subsequent changes in respective diets were introduced later, these nutritional guidelines are still applicable today and provide a global standard for purified mouse diets [19].

In line with these efforts, small size feed suppliers or larger companies accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) or other councils produce and/or market standardized mouse diets with fixed formulation, which allow reducing the fluctuation in study results (Table 1).

Table 1. Representative producers of mouse chows.

Company	Principal Office	Homepage
Research Diets, Inc.	New Brunswick, NJ, USA	https://www.researchdiets.com/
Ssniff Spezialdiäten GmbH	Soest, Germany	http://www.ssniff.com/
Altromin Spezialfutter GmbH & Co. KG	Lage, Germany	https://altromin.de/
BioServ	Flemington, NJ, USA	https://www.bio-serv.com/
Envigo Teklad (formerly Harlan Teklad)	Madison, WI, USA	https://www.envigo.com/
CLEA Japan, Inc.	Tokyo, Japan	https://www.clea-japan.com/
Specialty Feeds	Glen Forest, Western Australia, Australia	http://www.specialtyfeeds.com
Safe	Augy, France	www.safe-diets.com/
LabDiet	St. Louis, MO, USA	https://www.labdiet.com/
TestDiet	St. Louis, MO, USA	https://www.testdiet.com
Dyets, Inc.	Bethlehem, PA, USA	https://dyets.com/
Special Diets Services (SDS)	London, UK	http://www.sdsdiets.com/

4. Pasteurization

Pasteurization is a term named for the French scientist Louis Pasteur for a mild heat-treatment process used to destroy pathogenic microorganisms and preventing of spoilage of foods and beverages. For pasteurization of milk, for instance, a low-temperature, long-time process (LTLT) for 30 min heating at 63 °C or a 15 sec high-temperature short-time process (HTST) is used to inactivate large (but not all) spoilage-causing vegetative forms of microorganisms [21]. Beside LTLT and HTST, very short heating to 138 °C or above for at least 2 sec (ultra-pasteurization) is used for specialized applications [21].

Autoclaving referring to a process of sterilization under pressure is believed to be one of the most efficient methods of sterilization and common practice in many research institutions [22]. This method is inexpensive, convenient and guarantees the destruction of all microorganisms including spores and viruses. Therefore, pasteurization is commonly used for "sterilization" of mouse diets (Figure 4).

Usually, animal diets are autoclaved at 121 °C for 20 min, which can result in pellet hardness resulting in a significant reduction in wastage and in apparent and true consumption of the pelleted diet [23]. In addition, losses of pantothenate, vitamin A and vitamin D were found during autoclaving, while thiamine, riboflavin and pyridoxine were less affected [24]. In particular, autoclaving at higher temperatures for shorter period were more detrimental than autoclaving for longer time intervals at

lower temperatures [24]. Therefore, feed fortification with vitamins is potentially necessary, especially after autoclaving at high temperatures to ensure that the maintained mice receive an adequate and balanced supply with these essential nutrients.

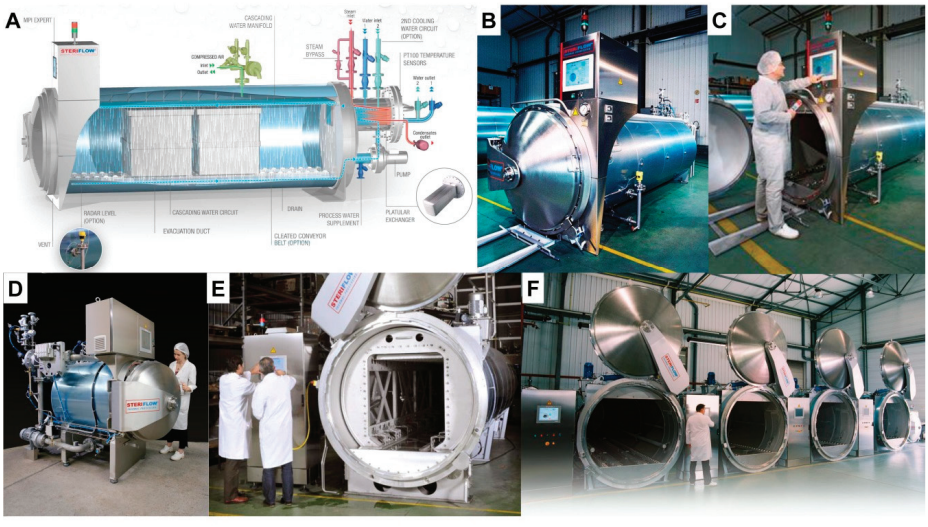

Figure 4. Sterilization of mouse diets by autoclaving. (**A**) Industrial autoclaves that can be used for sterilization of large batches of rodent diets must have large capacities. In principal, these devices are large pressure chambers, in which the goods are sterilized by subjecting them to a pressurized saturated steam at 121 °C (249 °F) maintained for about 20 min in a locked chamber. Different electronically-controlled valves and lines regulate steam flow and temperature in the steam chamber. (**B**,**C**) A typical industrial computer-controlled autoclave is depicted. (**D**–**F**) Autoclaves used for food production can be highly variable in size. Images showing different autoclaves from Steriflow (Roanne, France) were kindly provided by Kai Bergner from Vos Schott GmbH (Butzbach, Germany). A vivid 3D animation video of a typical water cascading process for sterilization by autoclaving can be found at: https://youtu.be/bWD87VVtzKU.

In addition, it is known that this procedure exerts undesirable effects on feed quality due to production of toxic compounds (e.g., acrylamide) and reduction of the overall nutritional value [22]. The autoclaving of a standard rodent diet resulted in a 14-fold increase in acrylamide, while the content of endogenous acrylamide in diets subjected to irradiation was reduced [25]. The forming of acrylamide is strongly correlated to the temperature used for sterilization [22,25]. In addition, autoclaved food products with high quantities of acrylamide produce elevated concentrations of epoxides, which are highly reactive chemicals, acting as mutagens [22]. Therefore, investigators and institutions should consider the detrimental and toxic effects that autoclaving might provoke in mouse diets.

5. Irradiation

In some cases, the diets are irradiated with γ-rays to eliminate remaining microorganisms residing in the feed. The microbial reduction strategies are often used to sterilize diets used for animals kept under SPF conditions [26]. In a typical sterilization process, the required dose depends on the "initial bioburden" and irradiation doses of between 20 and 30 kGrays (kGy) are used most frequently to treat diets intended for SPF animals, while larger doses (40–50 kGy) are recommended for diets intended for gnotobiotic or germ-free animals [26]. The physical unit Gy is defined as the absorption of one J of radiation energy per kg of irradiated material. For irradiation of large quantities of mouse

diets, the pallets of product are loaded onto conveyors moving around the γ-ray source. In most of the commercial facilities for food irradiation, cobalt-60 (^{60}Co) is the most common source of γ-rays. The respective facilities contain a number of safety systems, which are designed to avoid exposure of personnel to radiation. Furthermore, such irradiation devices are girded by a thick shield that hampers the penetration of γ-rays to the outside (Figure 5).

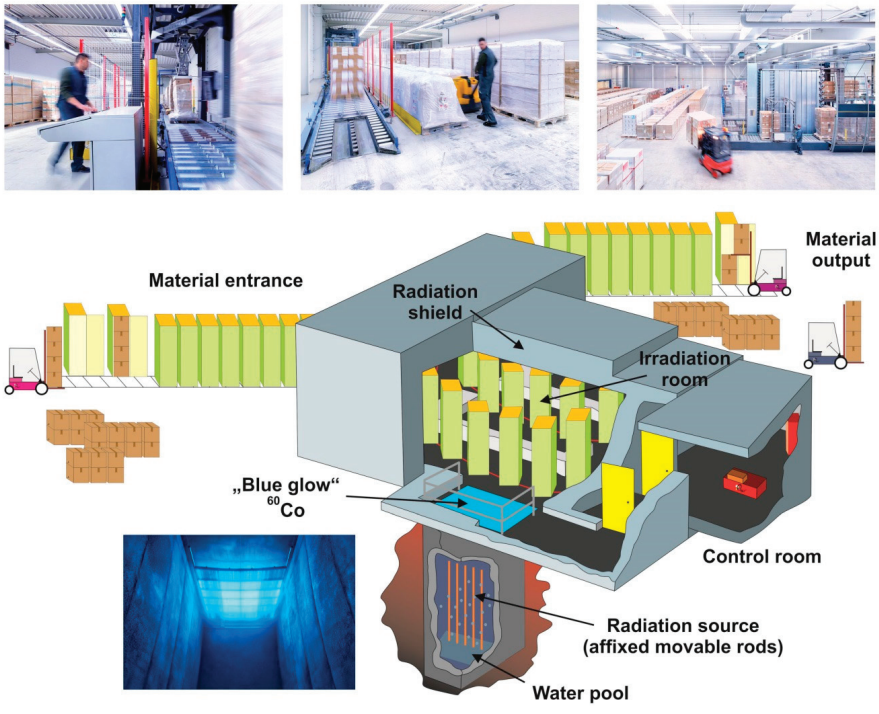

Figure 5. Irradiation of large batches of mouse diets. To minimize the risk of diet spoilage by pathogenic organisms, diets can be exposed to ionizing radiation. For irradiation in large scale, the packed diets are most commonly irradiated with γ-rays from a cobalt-60 (^{60}Co) source that has high penetration depth and dose uniformity and is able to penetrate relatively dense products. When not in use, the radiation source is stored in a water-filled storage pool, which absorbs the radiation energy. This Cherenkov radiation results in a blue appearance of the water bath, which is commonly known as "blue glow". For radiation, the ^{60}Co rods are lifted out from this pool and the emitted energy is directed to the goods to be irradiated or the goods are moved around the γ-ray source. The facility is surrounded by a thick concrete wall to avoid radiation leakage into the environment. The photos of the irradiation facility and the blue glow were kindly provided BGS Beta-Gamma-Service GmbH & Co. KG (Wiehl, Germany, ©BGS/M. Steur).

Although it is often argued that the doses used to destroy microorganisms are rather low, caution is advised because γ-rays at these doses have profound effects on some the integrity of the individual ingredients of the diet. This was impressively shown in a systematic study, in which the amounts of fat, protein, carbohydrate, and vitamins was investigated, showing that γ-rays at a dose of about 30 Gy have profound and selective effects on the stability of vitamin A and peroxide content of dry animal diets [26]. In line, a more previous study summarizing the main finding of published literature showed profound losses of the vitamins C, B_1, E, and A in food after its irradiation [27]. Moreover, destruction of highly polyunsaturated fatty acids up to 98% and destruction of fatty acids with two double bonds

up to 46% with accompanying lipid peroxide formation after irradiation with doses of 2–10 kGy [28], in which the effective dose of γ-rays refers to the amount of radiation that penetrate in the middle of the product to be irradiated.

6. General Remarks on the Origin of Nutrients in Purified Diets

In nature, mice have an extremely diverse diet, consuming practically any food source to which they have access. In order to be fed up, wild mice spend many of their active hours (~20 h) searching for these food products. Contrarily, the amount of time taken for a laboratory mouse to gnaw and eat well-balanced food directly from the cage hopper is considerably less [29]. Ideally, these preformed diet products are nutritionally either complete for various life stages from breeding through long-term maintenance, or adapted to special needs occurring during husbandry and housing. Global sold mouse diets contain a well-balanced mixture of proteins, carbohydrates, fats, vitamins, minerals, and potential additives that may be modified for the special needs of the biomedical research undertaken. In these formulations, substances that have been reported to have adverse confounding effects on experimental results or are toxic to the animals should as far as possible be omitted and should conform to the nutrient requirements of mice established by the National Research Council (see below). When properly stored at room temperature or cooler, depending on the composition of the diet, with ideally lower 50% relative humidity, these diets are usually stable for 6–12 months. To prevent continuous exposure to light and air, the storage in the original packaging or closed containers is recommended. Special diets enriched with temperature-sensitive additives may require the storage at lower temperature (4 °C or −20 °C). To avoid contamination, the products should be stocked in a proper environment.

The individual substance classes of which a diet is composed may originate from different sources (Table 2). The respective source may have an impact on energy intake, feed efficiency, apparent nitrogen and fat digestibility, composition of gut microbiota, and of course, of body weight development in mouse [30].

Table 2. Typical purified ingredients for special nutritional requirements of diets.

Substance Class	Representative Compounds
Proteins	Casein, soy protein isolate, egg white protein; crystalline amino acids
Carbohydrates	Sucrose, fructose, corn starch, (nondigestable oligosaccharides)
Fats	Lard, corn oil, safflower oil, Menhaden oil; soybean oil
Vitamins	Vitamin A, vitamin D, vitamin E, vitamin K, vitamin B_{12}, biotin, choline, folates, niacin, pantothenic acid, vitamin B_6 (pyridoxine, pyridoxal, pyridoxamine), riboflavin, thiamine, vitamin C
Minerals	Dicalcium phosphate, sodium selenite
Fibers	Cellulose, guar gum, pectin, carboxymethylcellulose, carrageenan, xanthan gum, gum arabic, inulin, fructooligosaccharides
Additives	Genistein, daidzein, cholesterol, myo-inositol
Special additives	Doxycycline, tamoxifen

6.1. Proteins

Casein, soy protein isolate, egg white proteins often serve as sources for proteins in respective chows. Caseins are the most frequent protein constituent in animal milk from cow, sheep and buffalo containing with an intrinsically disordered structure forming large colloidal particles with calcium phosphate to form casein micelles [31]. It is enriched in proline, which distorts protein folding into α-helices and β-sheets preventing the formation of higher proportions of secondary and tertiary protein structures. However, casein proteins are important nutritionally because of their high phosphate content due to which they bind significant quantities of calcium ions [31].

Compared to casein, soy protein isolate has a hypocholesterolemic effect [32] due to a lower intestinal absorption of cholesterol, increased steroid excretion, and a greater biological activity in decreasing hepatic lipogenic enzymes [32]. As a consequence, mice fed soy protein isolate or soy protein isolate hydrolysate diets have lower body weight, lower plasma cholesterol and glucose levels compared to animals that are fed with a casein diet [32].

Egg white contains proteins high in amino acid balance, only low quantities of carbohydrate, and is almost free of fat [33]. Compared to other diets, the consumption of egg white in mice has no significant impact on total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), or triglyceride levels, and suppresses food intake, dietary fat absorption, and fat accumulation, thereby preventing the formation of glucose tolerance [34,35]. However, white egg supplementation is supposed to induce oral desensitization and immune tolerance in mice [36]. Nowadays, egg white as a protein source is not often used. Nevertheless, this protein source can be helpful in studies analyzing effects of zinc-deficient diets. This is due to the fact that the zinc-binding capacity of casein is about 8.4 µg–30 µg/mg casein [37,38], while the maximal amount of bound zinc in egg white products is estimated to be only 1.3–1.6 ng/mg [39]. Moreover, egg white contains large quantities of the anti-nutrient avidin having strong affinity for biotin, preventing its absorption across the gastrointestinal tract [40]. Therefore, supplementation with biotin is sometimes necessary when using egg white as the major protein source.

Chemically-defined diets containing crystalline amino acids as the sole source of nitrogen as an alternative to complete proteins have also been successfully used for mouse maintenance [41]. It has been shown already decades ago that amino acid rations, when properly compounded, will provide a rate of growth in mice closely approaching that obtained with casein [42].

6.2. Carbohydrates

It is well-known that mice become obese when offered free access to sugars, but it is not established whether specific sugars are more likely to cause DIO [43]. Most common in purified diets as sugar sources are sucrose, fructose, and corn starch. The disaccharide sucrose is a disaccharide composed of glucose and fructose produced naturally in plants and after oral uptake efficiently hydrolyzed by sucrose in the intestinal mucosa to its constituent monosaccharides [44]. Free glucose elicits a glycemic and insulinemic response that stimulate the uptake of this sugar into cells, while fructose is mainly metabolized in hepatocytes via insulin-independent mechanisms not regulated by energy supply [44]. Sucrose has an energy of 16.8 kJ (4 kcal) per gram. Interestingly, sucrose stimulates higher daily intakes than isocaloric fructose solution in mice [43]. In animals, the fruit monosaccharide fructose produces profound metabolic disturbances, including insulin resistance, impaired glucose tolerance, high insulin and triglyceride levels, hypertension, dyslipidemia, and microvascular hepatic steatosis [45]. However, the susceptibility to sugar-induced obesity varies with strain [45]. It has an energy density of 15.75 kJ/g (3.75 kcal/g) and feeding mice with a high fructose diet induces hepatic lipid accumulation by activating lipogenic gene expression and de novo lipogenesis [46]. Therefore, this sugar is supposed to be one of the key dietary catalysts in the development of non-alcoholic fatty liver disease [47]. Interestingly, elevated uptake of fructose in mice can result in dysbiosis, increased hepatic lymphocyte infiltration, and further inflammation of gut, liver and fat tissue [47].

Corn starch also known as "cornflour" is a glucose polymer, highly branched carbohydrate (e.g., the starch) derived from the endosperm of the kernel of corn (maize) grain. In its pure form it is a tasteless, odorless, and cold water insoluble powder. In the body, starch is hydrolyzed by amylases into its constituent sugars. Its energy content is about 15.95 kJ/g (3.8 kcal/g). Depending on its formulation, certain glucose polymers may resist digestion in the small intestine in mammals and arrive in the colon where they will be fermented by the gut microbiota resulting in a large variety of products including short chain fatty acids (acetate, propionate, butyrate) that provide as a prebiotics a range of physiological benefits [48]. This should be critically kept in mind when performing experimental studies analyzing the impact and composition of gut microbiota on energy homeostasis, development

of obesity and its metabolic consequences [49]. Similarly, the feeding of C57BL/6J mice with HFD supplemented with resistant starch derived from maize resulted in an altered gut bacteria composition and corroborated with a significant shift in the liver metabolome [50].

6.3. Fats

A normal rodent diet contains about 10 kcal% fat, while diets enriched with 30–60 kcal% are defined as HFD, provoking significant weight gain and insulin resistance [51]. Typical fat constituents in mouse diets are lard, corn oil, safflower oil, or Menhaden oil.

6.3.1. Lard

Lard is a semi-soft fat derived from adipose tissue of the pig and contains a high content in saturated fatty acid (~30%) and <1% trans-unsaturated fatty acids (i.e., trans fats). In some cases, lard is hydrogenated or treated with bleaching and deodorizing agents, emulsifiers, and antioxidants to improve its stability. The energy content of lard is about 37.6 kJ/g (9 kcal/g). Interestingly, in rodents lard-based HFD accentuated the increase in weight gain and the development of obesity and insulin resistance more than a diet that was based on hydrogenated vegetable-shortening diets, suggesting that the outcome of consuming HFD is strongly dependent on the used fat constituent [52]. Moreover, lard-based diets were significantly more inferior than soybean oil in protecting mice after application of the powerful hepatotoxin carbon tetrachloride twice a week for three weeks, which is a model to generate liver necrosis and steatosis, potentially indicating its less antioxidant activity [53].

6.3.2. Corn Oil

Refined corn oil is derived from the germ of maize and typically contains 99% triacylglycerols with 59% polyunsaturated fatty acid (e.g., linoleic acid), 24% monounsaturated fatty acid (e.g., oleic acid), and 13% saturated fatty acid (e.g., palmitic acid, stearic acid, arachidic acid) [54,55]. It is categorized as one of the richest sources of health-promoting phytosterols and tocopherols protecting against DNA damage, hypertension, platelet aggregation, hypercholesterinemia, and diabetes [54]. In line with these beneficial effects, high corn oil dietary intake was shown to improve health and longevity of aging mice when fed at normal energy balance [56]. In addition, disease development and progression as well as deposition of extracellular matrix within the liver in a mouse model of non-alcoholic steatohepatitis (NASH) was significantly reduced when the HFD was composed of corn oil instead of non-trans fats [57].

As mentioned, the dietary intake of corn oil is known to improve health and longevity of mice, which corroborates with reversing aging-increased blood lipids and decreasing serum pro-inflammatory markers [56]. In addition, the olfactory cues and the oily texture of corn oil are important orosensory factors provoking a strong appetite in mice [57]. However, other reports showed that the excess dietary intake of polyunsaturated fatty acids is associated with loss of spontaneous physical activity and development of insulin resistance [58]. In addition, polyunsaturated fatty acids are subject to oxidation. Therefore, the AIN recommended the supplementation of antioxidants in formulations containing large quantities of corn oil [19].

6.3.3. Safflower Oil

The safflower (*Carthamus tinctorius*) or safflor is a thistle-like annual plant of the *Asteracea* family from which vegetable oil can be extracted from its seeds. Safflower seed oil is flavorless and colorless and in its composition similar to oil from sunflowers, olives, and peanuts, typically containing high content of linoleic acid (63–72%), oleic acid (16–25%) and linolenic acid (1–6%) [59]. In particular, the high content of linoleic acid was shown to have highly beneficial health-promoting effects by reducing the expression of lipogenic enzymes and increasing the activity of hepatic fatty acid oxidation enzymes [60].

6.3.4. Menhaden Oil

The forage fish menhaden (*Brevoortia tyrannus*) belongs to the herring family and forms large flocks occurring on the North American Atlantic coast from Nova Scotia to Florida and related forms are also found up to the coasts of Argentina. The oil derived of these animals is rich in omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPAc) and docosyhexaenoic acid (DHAc), both supposed to have anti-inflammatory activities [61]. Recently, it was demonstrated that EPAc and DHAc supplementation in the context of HFD partially mitigated reductions in insulin sensitivity and maintaining cell function [62]. Moreover, the polyunsaturated fatty acids in menhaden oil prevented high-fat diet-induced fatty liver disease in mice [63].

6.4. Vitamins

Mice, like humans, require some essential micronutrients in small quantities that cannot be synthesized by their own. These vitamins are organic molecules and must be obtained through the diet, or alternatively synthesized by microorganisms in the gut flora. They have diverse biochemical functions and are commonly sub-classified as either water-soluble (vitamin C, vitamin B_1, vitamin B_2, vitamin B_3, vitamin B_5, vitamin B_6, vitamin B_7, vitamin B_9, vitamin B_{12}) or fat-soluble factors (vitamin A, vitamin D, vitamin E, vitamin K). In comparison to fat-soluble vitamins that can accumulate in the body, water-soluble vitamins are readily excreted from the body. Deficient intake (primary deficiency), malfunction during absorption or use of a vitamin (secondary deficiency), or increased consumption results in hypovitaminosis. In contrast, excess intake results in hypervitaminosis occurring mainly only with fat-soluble vitamins (e.g., vitamin A and D). The different vitamins are involved in many biochemical processes (Table 3). Therefore, any shortage might result in complex illnesses potentially affecting different organs. General guidelines defining the nutrient requirements of the mouse are available (see below) [10].

Table 3. Biochemical function of water-soluble and fat-soluble vitamins.

Vitamin Sub-Class	Vitamin	Compound (Alternate Names)/Members	Biochemical Function	Reference
Water-soluble	Vitamin C	Ascorbic acid	Maintenance of redox balance; co-substrate for several enzymes; intracellular antioxidant; electron donor	[64]
	Vitamin B_1	Thiamine	Coenzyme in the catabolism of sugars and amino acids	[65]
	Vitamin B_2	Riboflavin	Coenzyme in flavoprotein enzyme reactions (e.g., FAD); antioxidant	[66]
	Vitamin B_3	Niacin (nicotinic acid)	Coenzyme involved in protein, fat and carbohydrate metabolism (e.g., Nicotinamide adenine dinucleotide (NAD); hydrogen carrier; antioxidant, reducing agent	[67]
	Vitamin B_5	Pantothenic acid	Coenzyme in synthesis and metabolism of proteins, carbohydrates and fats; required for synthesis of coenzyme A	[68]
	Vitamin B_6	pyridoxine, pyridoxal, pyridoxamine and their respective mono-phosphorylated derivatives	Coenzyme in many enzymatic reactions (decarboxylations, transaminations, eliminations, racemizations, transsulfurations, interconversions); antioxidant	[69]
	Vitamin B_7	Biotin (vitamin H)	Coenzyme for carboxylases: pyruvate carboxylase, 3-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, and coenzyme for acetyl-CoA carboxylase 1 and 2	[70]
	Vitamin B_9	Folic acid (folacin), folate pteroyl-L-glutamic acid, pteroyl-L-glutamate, pteroylmonoglutamic acid	Coenyzme in single-carbon group (methyl-, methylene-, formyl group) transfer reactions	[71]
	Vitamin B_{12}	Cobalamin	Coenzyme for the methionine synthase and methylmalonyl-CoA mutase	[72]

Table 3. *Cont.*

Vitamin Sub-Class	Vitamin	Compound (Alternate Names)/Members	Biochemical Function	Reference
Fat-soluble	Vitamin A	Retinol, retinal, retinoic acid, provitamin A carotenoids	Modulator of immune system; low-light and color vision; wound healing; hormone (binding to retinoic acid receptors); metabolic effects; reproduction	[73,74]
	Vitamin D	Group of secosteroids (e.g., ergocalciferol, cholecalciferol and others);	Binding to vitamin D receptor acting as a transcription factor; calcium and phosphate homeostasis; immune system; cell proliferation and differentiation; bone formation; innate and adaptive immunity	[75,76]
	Vitamin E	α/β/γ/δ-tocopherols, α/β/γ/δ-tocotrienols	Antioxidant and radical scavenger; modulator of gene expression; enzyme activity regulator (e.g., protein kinase C)	[77]
	Vitamin K	Phylloquinone (vitamin K1), menaquinones MK-4 through MK-10 (vitamin K2)	γ-glutamyl carboxylation; relevant in blood coagulation and bone metabolism; modulator of transcriptional activity; agonist of steroid and xenobiotic nuclear receptor; neural stem cell differentiation modulator	[78]

Based on the heterogeneous character of the different vitamins, their stability is highly variable. Quantitatively deterioration in content over time of vitamins can be affected by many factors, including temperature, moisture, oxygen, light, pH, oxidizing and reducing agents, catalytic activity of metals, mutual damage by other vitamins, detrimental compounds (e.g., sulphur dioxide), or combination of these factors [79]. For example, vitamin B_{12} is decomposed by light, alkali, acids, and oxidizing or reducing agents, while on the contrary vitamin B_2 (riboflavin) and vitamin B_3 (niacin) are rather stable [79].

In addition, during production and handling of mouse diets, there are several factors affecting the stability of vitamins during extrusion. These occur for example during handling of raw material, mixing, conditioning, processes, changes in moisture, heat or pressure treatments during extrusion and expansion [80]. Aspects of stability of vitamins and reduced levels of vitamins during processing of fish feed were concisely discussed by Riaz and coworkers [80]. Since the production of grain-based diets is rather similar, the reported values should be comparable with these values. For the different vitamins, the factors affecting vitamin destruction during processing and storage are different (Table 4). Sufficient supply with vitamins in mouse diets can be guaranteed by food fortification.

Table 4. Vitamin losses during pelleting, extrusion and storage of feeds and factors affecting vitamin deterioration.

Vitamin	Factors Affecting Vitamin Stability during Processing and Storage
Vitamin C (ascorbic acid)	Moisture, heat, oxidation, light, iron
Vitamin B_1 (thiamine)	Oxidation
Vitamin B_2 (riboflavin)	Light
Vitamin B_3 (niacin)	Rather stable
Vitamin B_5 (pantothenate)	Oxidation, light
Vitamin B_6 (pyridoxine)	Oxidation, reduction
Vitamin B_7 (biotin)	Oxidation
Vitamin B_9 (folic acid)	Oxidation, light, microbial
Vitamin B_{12} (cobalamin)	UV light, interaction with other water-soluble vitamins, heat, pH
Vitamin A (retinol, retinal, retinoic acid, provitamin A carotenoids)	Oxidation, light, (trace elements)
Vitamin D (cholecalciferol)	Stable
Vitamin E (α/β/γ/δ-tocopherols, α/β/γ/δ-tocotrienols)	Oxidation, light, oxidized fat
Vitamin K (menadione)	Oxidation

Information of this table was taken in simplified and modified form from [80] and complemented with data from [79,81].

6.5. Minerals and Trace Elements

Minerals, also known as macrominerals or micronutrients, are inorganic elements which generally occur in large quantities, while trace elements or microminerals normally are present only in small amounts in organisms. Calcium, phosphorus, chloride, magnesium, phosphorus, potassium, sodium, and chloride are the elements playing vital roles in the body [82]. They regulate the proper composition and function of the body fluids, tissue, bone, teeth, muscles and nerves. Some of them also have function as a coenzyme in metabolic reactions and guarantee biochemical functions in body homeostasis, including energy production, growth, wound healing and proper utilization of vitamins and other nutrients. Essential trace elements required in smaller amounts by animals and plants are iron, zinc, copper, nickel, molybdenum, manganese, selenium, iodine, and others. These elements are involved in vital enzymatic reactions by acting as cofactors or by stabilizing cellular structures [82]. Based on their involvement in hundreds of biological processes, inadequate mineral and trace element intake can result in severe health conditions that can affect nearly all organs and tissues. These can be highly variable and become most evident after chronic shortage (see below).

6.6. Fibers

In its simplest definition, fibers are non-starch polysaccharides composed of a large number of monosaccharides that are linked through covalent bonds [83]. The term "dietary fibers" is often used to designate the sum of non-starch polysaccharides with its complex fibrous, tasteless organic polymers (i.e., the lignin) forming key structural materials in the supportive tissue of plants of which it is derived of [83]. Based on its composition, these dietary fibers have different physiochemical properties regarding size, hydration, viscosity, fermentability, and impact on satiety. In addition, the proportion of the cell wall components varies from plant to plant and is further dependent on the age and type of plant tissue.

Dietary fibers are roughly grouped into soluble and insoluble fibers. Soluble fibers (non-cellulosic polysaccharides, arabinoxylans, β-glucans, some hemicelluloses, pectins, gums, mucilages, inulin) dissolves in water and are broken down in the gut some of which form a thick, spread-out gel, while insoluble fibers (cellulose, some hemicelluloses, lignin, resistant starch) are left intact as food moves through the gastrointestinal tract [84]. Some soluble fibers block the uptake of fats and are used as a fermentable energy source for gut bacteria. On the contrary, insoluble fibers are indigestible and speed up the elimination of toxic waste in the digestive tract through promoting bowel movement in the colon, thereby preventing constipation.

Fibers can be further sub-classified as neutral detergent fiber (NDF) and acid detergent fiber (ADF), in which NDF is the complete fraction of insoluble residue following neutral detergent digestion and ADF is the harder to digest part of the fiber. In other words, ADF is the sum of cellulose and lignin and NDF is the sum of ADF and hemicellulose. ADF is the fraction of fibers that contain virtually no fermentable ability and reduces overall digestible energy from the diet and NDF is a measure of most of the fiber in the diet (except for soluble fiber, which is not part of this fraction). Therefore, a high content of ADF in a diet will provide lower amounts of energy than a diet with lower ADF amount [85].

There are a large number of fiber sources used to dilute the nutrient and energy density of the diets (cf. Table 2). When fibers are included in rodent chows, the weight of the cecum and colon may increase and microbial fermentation results in short-chain fatty acid (SCFA) production such as acetate, propionate and butyrate having beneficial effects on mice health [86,87]. Moreover, addition of fibers that dilute the nutrient density of the diet will have effects on food intake and body weight and further impact the fecal and urinary nitrogen excretion as a result of microbial fermentation [10]. In humans, diets with a high content of fibers are suggested to have beneficial effects, including increasing the volume of fecal bulk, decreasing the overall time used for intestinal transit, promoting the elimination of toxic waste, stimulating the intestinal flora, and finally reducing the onset risk of metabolic syndromes (Figure 6) [88]. Comparable to humans, a low-fiber diet was shown to promote expansion and activity of colonic mucus-degrading bacteria, suggesting respective diets are ideally

suited as nutritional models for analyzing aspects of colonic mucus layer dysfunction and altered pathogen susceptibility [89].

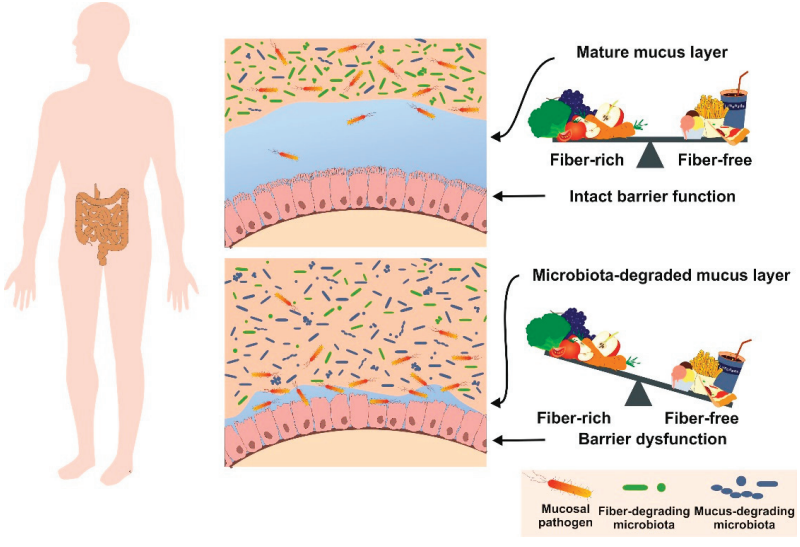

Figure 6. Beneficial effects of dietary fibers in humans. The ingestible parts of plants help to speed up the elimination of toxic waste in the digestive tract through promoting bowel movement in the colon and reacting with bacteria in the lower colon, thereby producing short chain fatty acids (acetate, propionate, butyrate) causing cancer cells to self-destruct. Inadequate fiber intake during malnutrition results in distortion of the mucosa, reduced intestinal barrier function, and inflammation. Similarly, fiber-free diets provoke degradation of mucus layer and barrier dysfunction in mouse.

7. Nutrient Requirements of the Mouse

Mice as humans need a balanced, fresh and healthy diet that meets their nutritional needs. Nutrients designed for rats, guinea pigs, hamsters or other herbivores are not necessarily suitable for mice, because they need sufficient quantities of essential amino acids, fatty acids, vitamins, and minerals that might vary in content to other animals.

When housed under a standard 12-h light/12-h dark cycle, mice typically consume the majority of their food during the dark period, with short bouts of feeding during the light period [90]. There are a number of factors impacting food uptake, including strain differences, genetic background in transgenic and knockout mice, age, stress, habituation, forced movement, and discomfort resulting from drug treatments or surgeries. Moreover, the energy balance in female mice is strongly affected by hormonal variation associated with the estrous cycle [90].

Detailed guidelines for the nutrient requirements of laboratory animals were first published in 1962 and updated several times [10]. In the most recent edition, published in 1995, the composition of an adequate nutrition of mice maintained in conventional animal facilities is in detailed listed (Table 5). It should be noted that mice kept in a germ-free SPF facility or subjected to experimental-induced stress have altered nutrient requirements that should be adapted accordingly.

Table 5. Nutrient requirements of mice maintained in conventional animal facilities *.

Nutrient	Amount (per kg Diet)
Lipid	50 g
Linoleic acid	6.8 g
Protein (N × 6.25) **	180–200 g
Amino acids	Arginine: 3 g; histidine: 2 g; isoleucine: 4 g; leucine: 7 g; valine: 5 g; threonine: 4 g; lysine: 4 g; methionine: 5 g; phenylalanine: 7.6 g; tryptophan: 1 g
Minerals	Calcium: 5 g; chloride: 0.5 g; magnesium: 0.5 g; phosphorus: 3 g; potassium: 2 g; sodium: 0.5 g; copper: 6 mg; iron: 35 mg; manganese: 10 mg; zinc: 10 mg; iodine: 150 µg; molybdenum: 150 µg; selenium: 150 µg
Vitamins	Retinol: 0.72 mg (= 2400 IU); cholecalciferol: 0.025 mg (1000 IU); RRR-α-tocopherol: 22 mg (= 32 IU); phylloquionone: 1 mg; biotin: 0.1 mg; choline: 2 g; folic acid: 0.5 mg; niacin: 15 mg; Ca-pantothenate: 16 mg; riboflavin: 7 mg; thiamine-HCl: 5 mg; pyridoxine-HCl: 8 mg; cobalamin: 10 µg

* The information depicted for individual nutrients was taken from the National Research Council (NRC) guidelines [10]. According to these guidelines, the nutrient requirements are expressed on an as-fed basis for diets containing 10% moisture and 16–17 kJ metabolizable energy per g and should be adjusted for diets of differing moisture and energy concentration. ** This calculation of this parameter assumes that the average nitrogen (N) content of proteins is about 16 percent, which led to the use of the calculation N × 6.25 (1/0.16 = 6.25) to convert nitrogen content into protein content. The amount of protein is given for animals maintained under regular growth conditions.

It was demonstrated some years ago that the composition of the commensal gut microbiota in humans correlates with diet and health in the elderly [91]. Moreover, in aged mice some of the alterations associated with aging can be rescued by fecal transfer [92]. In this context, it should be noted that the eating of fresh feces, which is a natural behavior of mice, is possibly not only helpful to better absorb nutrients/minerals they need to stay healthy, but is further a requirement to slow down the aging processes caused by nutritional deficiencies. The consequences of a chronic shortage in a specific nutritional compound have been best documented in mouse studies in which diets were fed lacking individual substances. Such studies have shown that the permanent lack in a specific component evokes severe consequences (Table 6).

Table 6. Consequences of insufficient supply with selected nutritional components.

Component	Consequences of Insufficient Supply	References
Fat	Alterations in composition and functionality of synaptosomal plasma membranes	[93]
Protein	Decreased host immune defense; reduced numbers of splenocytes, lower quantities of glutathione in several organs; less food ingestion and weight gain during pregnancy resulting in fewer viable pups	[94,95]
Phe, Thr, Trp, Met, Lys, Leu, Ile, Val	Massive reduction of abdominal fat mass after 7 days, most likely via increased energy expenditure	[96–99]
Calcium	Development of osteoporosis-like symptoms with reduced femur length and reduced density of various bones	[100]
Magnesium	Hypomagnesemia reduced bone growth and chondrocyte functionality; During embryogenesis embryotoxic effects (retardation, disturbed bone development and skeletal malformations) are induced	[101,102]
Phosphorus	Severe growth retardation with a 50% reduction in body weight; reduced formation of milk droplets	[103,104]
Potassium	Hypokalemia results in decrease in luteinizing hormone and testosterone provoking testicular impairments that is also seen in a fall in the weight of seminal vesicles	[105]
Copper	Development of anemia, duodenal hypoxia and alterations in intestinal iron absorption	[106]
Iron	Development of pronounced splenomegaly; anemia with reduction in hemoglobin and hematocrit levels with higher risk of developing deep dental caries	[107,108]

Table 6. *Cont.*

Component	Consequences of Insufficient Supply	References
Manganese	Growth retardation and malfunction of reproductive organs and ovulation; congenital debility of young animals with loss of both equilibrium and coordination	[109,110]
Zinc	Reduced immune responses after challenging with pathogen indicated by greater weight loss, stool shedding, mucus production and diarrhea	[111]
Iodine	Development of carcinoma of the thyroid; formation of oxidative stress and DNA modifications	[112,113]
Selenium	Potentiate the development of autoantibodies; reduction of amino acid levels and elevation of mononucleotides resulting in dysregulated metabolomes and age-associated decline of protein synthesis; development of widespread pyogranulomas	[114–116]
Thiamine	Reduction of energy state in the liver; reduction of blood glucose, insulin, triglycerides, cholesterol, liver glycogen; increase of serum lactate	[117]
Biotin	Impairment of mitochondrial structure and function; intoxication with propionyl-CoA; systemic inflammation	[118]
Vitamin D	Increased expression of sex steroid receptors in myometrium; increased expression of proliferation-related genes; promotion of fibrosis, inflammation, and immunosuppression; enhancement of DNA damage; increased lipid deposition in skeletal muscle and muscle fiber disorganization	[119,120]
Vitamin A	Breakdown of oral tolerance; reduction of iron absorption	[121,122]
Linoleic acid	Reduction results in lower concentrations of circulating, small bowel and hepatic endocannabinoids; lower feed efficiency and weight	[123]
Choline	Amplification of liver fat accumulation in phases of high fat consumption; lowering of fasting plasma insulin; improvement of glucose tolerance; reduction of fibroblast-like cells in circulating tumor cells and less metastasis	[124,125]

The resulting phenotypes resulting from chronic shortage in specific elements or compounds can vary dramatically. The nutritional status impacts growth, reproduction, longevity and determines the response to pathogens, environmental stress, and organ function. Therefore, the avoidance of inadequate intake is one important factor in guaranteeing the welfare of the mouse kept in an animal facility.

8. Representative Examples of Diet-Induced Obesity and Fatty Liver Disease

The composition of a diet has strong impact on the health of an organism. It influences the composition of the gut microbiota and overfeeding or fasting can cause disease. Therefore, scientists frequently use such model to analyze aspects of diet-related diseases. On the contrary, lifelong caloric restriction is an effective experimental tool to reprogram hepatic fat metabolism and to extend life span in diverse species [126].

In biomedical research, mice are the most widely used animals and the nutrient requirements might depend on development state, reproductive activity, age, and stress factors induced by the experimental conditions. Moreover, there is a great danger that mice, when housed in standard laboratory under ad libitum feeding conditions having continuous access to food but virtually no environmental stimulation become overfed and sedentary and are potentially not suitable as proper controls in animal experiments [127]. Similarly, unwanted contaminants such as pesticides, mycotoxins, heavy metals, nitrosamines, nitrates, nitrites, phytoestrogens with estrogenic activity, and polychlorinated biphenyls in dietary products may affect the outcome of animal studies when present at a sufficient high concentration [128]. Maximum allowable concentrations of these undesirable substances in mouse diets are also specified by the guidelines provided by organizations such as the US Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), the British Association for Research Quality Assurance (BARQA), or the Society of Laboratory Animal Science, GV-SOLAS [128].

Therefore, depending on the research question, the nutritional requirements must be carefully considered. Contrarily, the usage of specific diets in mice is widely applied to induce diseases mimicking

human pathologies including liver disease, metabolic dysfunctions (insulin resistance, diabetes type 2), heart failures, immune system alterations, neurological disorders, or even cancer. These diet-induced models are enriched in specific fats, sugars, toxins, metals, or alternatively lack essential nutrients that are indispensable for the proper synthesis of essential nutrients. In particular, studies analyzing aspects of the immune system require special needs in regard to sterility and compounds that might interfere with the composition of the gut microbiome or function.

In most countries, the feeding of diets provoking the formation of disease or animal suffering require permission of responsible animal welfare authorities and should be carried out in an ethical framework that minimize fear, pain, stress and suffering of animals. This is best done, when respective experiments are carried out following established SOPs providing details about the scientific background, its implementation, experimental details (handling, concentrations, duration of procedure, biometric aspects, readout systems), and about the animal burden associated with this procedure [4]. The diversity of mouse diets is extremely versatile. The diets might vary in size, form, color and of course in nutritional composition (Figure 7).

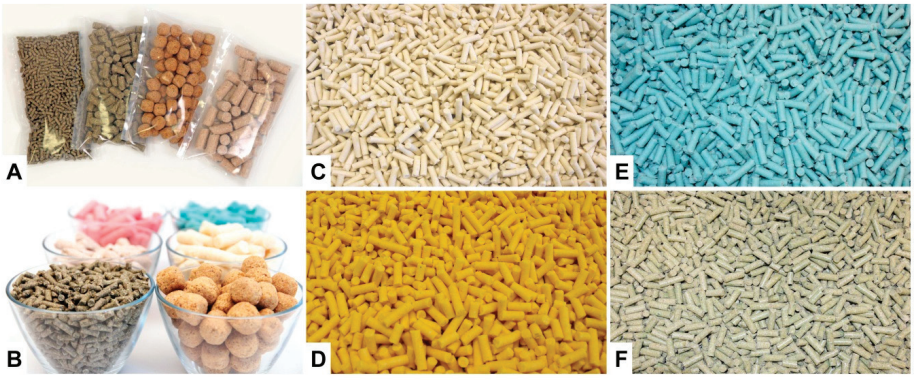

Figure 7. Diversity of mouse diets. (**A**) Diets can be produced in different appearance. (**B**) Diets can be produced in variable shape and color. (**C**) Uncolored diet, (**D**) yellow-stained diet, (**E**) blue stained high-fat diet, and (**F**) doxycycline hylate added diet. These figures were kindly provided by Dr. Dr. habil. Annette Schuhmacher (Ssniff Spezialdiäten GmbH, Soest, Germany).

Nowadays, a large number of diet-induced disease models are established in biomedical research. Most common are models to induce atherosclerosis, obesity, diabetes type 2, or liver damage. In the following, we will discuss some aspects of representative diets used in hepatology research, in particular, NAFLD. These are a typical control diet and four diets that are used to induce fatty liver injury, namely a diet to induce DIO, a typical Western diet (WD), a diet rich in fat, fructose and cholesterol (FFC), and the so called methionine-choline deficient (MCD) diet. Representative compositions of such diets are given in Table 7.

These four diets are some examples of diets commonly used in experimental hepatology research as nutritional models to induce a spectrum of disorders associated with accumulation of excess fat in the liver. The most common form is NAFLD and a more serious condition named non-alcoholic steatohepatitis (NASH). NAFLD and NASH and are the most prevalent liver diseases in Western society and the third leading cause for liver transplantation in the US [129]. Furthermore, there is evidence that NAFLD precedes and is associated with the metabolic syndrome characterized by obesity, diabetes, insulin resistance, and hypertension [130]. Phenotypically, patients with NASH/NAFLD are characterized by liver cell injury and damage, inflammation, and an increased risk for liver fibrosis and carcinogenesis [129]. Based on the eminent importance of NAFLD, several experimental dietary mouse models were developed to mimic the pathogenesis of human NASH and NAFLD.

Table 7. Composition of representative mouse diets used in hepatology research *.

	Component	Control Diet	DIO ** Diet	WD Diet	FFC Diet	MCD Diet
Crude nutrients [%]	Crude protein (N × 6.25)	17.6	24.4	17.3	19.7	15.0
	Crude fat	7.1	**34.6**	**21.1**	20.0	10.0
	Crude fibre	5.0	6.0	5.0	5.1	3.0
	Crude ash	3.2	5.3	4.2	4.4	3.3
	Starch	38.2	0.1	14.4	0.1	19.2
	Dextrin	**13.1**	**15.4**	0	**10.9**	0
	Sugar	11.2	9.4	34.3	34.2	45.1
	N free extracts	63.1	26.3	49.8	46.2	67.3
Minerals [%]	Calcium	0.55	0.92	0.76	0.78	0.59
	Phosphorus	0.37	0.64	0.45	0.50	0.46
	Sodium	0.15	0.20	0.24	0.20	0.16
	Magnesium	0.10	0.23	0.10	0.13	0.06
	Potassium	0.55	0.97	0.54	0.77	0.36
Fatty acids [%]	C 4:0	-	-	**0.80**	-	-
	C 6:0	-	-	**0.53**	-	-
	C 8:0	-	-	**0.29**	-	-
	C10:0	-	-	**0.63**	-	-
	C 12:0	-	**0.07**	**0.72**	0.02	-
	C 14:0	0.02	0.44	2.22	0.07	-
	C 16:0	0.84	7.93	5.60	2.04	1.09
	C 17:0	0.01	0	0.14	0.01	0.01
	C 18:0	0.26	4.37	2.05	2.38	0.19
	C 20:0	0.03	0.11	0.04	0.14	0.03
	C 16:1	0.01	0.94	0.38	0.08	0.01
	C 18:1	1.78	13.97	4.65	11.65	2.58
	C 18:2	3.69	4.64	0.38	2.14	5.53
	C 18:3	0.41	0.49	0.11	0.19	0.09
Amino acids [%]	Lysine	1.47	2.20	1.43	1.63	1.40
	Methionine	0.55	0.86	0.93	0.61	**0**
	Cystine	0.37	0.45	0.07	0.43	0.35
	Threonine	0.78	1.07	0.76	0.86	0.80
	Tryptophan	0.23	0.33	0.22	0.26	0.18
	Arginine	0.69	0.95	0.67	0.77	0.98
	Histidine	0.53	0.74	0.52	0.59	0.45
	Valine	1.23	1.70	1.20	1.37	0.81
	Isoleucine	1.00	1.38	0.97	1.11	0.80
	Leucine	1.75	2.42	1.71	1.95	1.10
	Phenylalanine	0.92	1.27	0.89	1.02	0.74
	Phenylalanine +Tyrosine	1.85	2.56	1.80	2.06	1.24
	Glycine	0.35	0.52	0.34	0.39	2.31
	Glutamic acid (+Glutamine)	3.97	5.50	3.88	4.43	3.96
	Aspartic acid (+Asparagine)	1.31	1.82	1.28	1.45	0.95
	Proline	2.02	2.80	1.97	2.25	0.35
	Serine	1.06	1.46	1.03	1.18	0.35
	Alanine	0.53	0.81	0.52	0.59	0.35
Vitamins [mg per kg]	Vitamin A ***	1.20	4.50	4.50	1.32	5.85
	Vitamin D_3 ****	0.025	0.0375	0.0375	0.0275	0.055
	Vitamin E	75	150	150	90	135
	Vitamin K (as MNB)	4	20	20	4	45
	Thiamine (B_1)	12	25	26	13	22
	Riboflavin (B_2)	16	16	16	18	22
	Pyridoxine (B_6)	7	16	16	7	22
	Cobalamin (B_{12})	0.025	0.03	0.03	0.028	0.03
	Nicotinic acid	29	47	49	32	98
	Pantothenic acid	15	55	55	17	60
	Folic acid	2	16	16	2	2
	Biotin	0.2	0.3	0.3	0.2	0.4
	Choline	1130	1140	920	920	**0**
Trace elements [mg per kg]	Iron	49	168	49	68	42
	Manganese	22	95	22	30	53
	Zinc	41	65	41	58	29
	Copper	10	13	11	14	6
	Iodine	0.3	1.2	0.3	0.4	0.2
	Selenium	0.2	0.2	0.2	0.2	0.1

Table 7. *Cont.*

	Component	Control Diet	DIO ** Diet	WD Diet	FFC Diet	MCD Diet
Other ingredients [mg/kg]	Cholesterol	0	~230	2070	20,000	0

* The concentration of the individual components were taken from Ssniff diets with order numbers E15712 (control), E15742 (DIO), E15721 (Western diet), E15766-3402 (NASH diet), and E15653 (MCD diet), respectively. ** Abbreviations used are: DIO, diet-induced obesity; FFC, fat-, fructose- and cholesterol-rich diet; HF, high fructose; MCD, methionine-choline deficient; MNB, menadione nicotinamide bisulfite; WD, Western diet. *** 1 mg Vitamin A corresponds to 3333 IU. **** 1 µg Vitamin D_3 corresponds to 40 IU. Percentages [%] are given in relation to the whole weight of the diet. Underlined values mark special features in the respective diet.

When comparing a diet used for DIO with a control diet, the most striking difference is the high-fat content of the DIO diet (cf. Table 7). Typically, mice fed a DIO diet containing 40–60% of calories from fat for 7–30 weeks increases their body weight and propensity to develop pre-diabetic symptoms and metabolic syndrome. This type of diet is, therefore, often used in studies investigating aspects of food intake, energy expenditure, glucose tolerance, insulin resistance, and elevated blood pressure [130,131]. When using this model, a slight increase in body weight can be noticed already after 2–4 weeks, while the body weight gradually increases thereafter and is 20–30% higher in mice after 16–20 weeks compared to chow-fed mouse [132]. However, the outcome of the DIO model is influenced by many factors, including genetic background, gender, age, and environmental factors such as cage placement, mice density, and mice handling [132].

While in a typical control diet, the fat and sugar content is not higher than 10%, a WD is characterized by a high content of fat combined with a high amount of a sugar as sucrose or fructose. In some cases, these diets are enriched with trace of SCFA such as C4:0 (butyric acid) and medium-chain fatty acids (MCFA) such as C6:0 (caproic acid), C8:0 (caprylic acid), and C10:0 (capric acid) are added to these diets. The rationale of this supplementation is the notion that ghrelin activation requires acetylation of its third residue, serine, with caprylic acid by ghrelin O-acyltransferase [133].

After prolonged feeding of a DIO diet or WD to mice for 30–50 weeks, the animals become severely obese, fat deposition occurs, ectopic fat accumulates in the body and the liver size significantly increases (Figure 8).

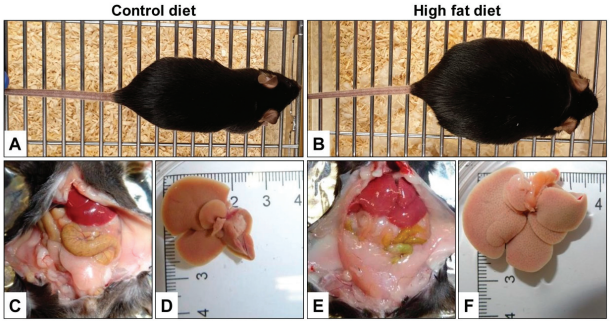

Figure 8. Diet-induced obesity and high-fat diets. (**A**,**B**) Comparison of mice receiving either a grain-based diet (**A**) or a diet enriched in fat (**B**) for prolonged times. While the body weight of the mice receiving a control diet was 25 g, the mouse fed a diet enriched for the same time was 52 g. (**C**–**F**) In obese animals, excess fat deposition and ectopic fat accumulation in the body occurs (**C**,**E**). In addition, the liver size is much higher in animals that received a diet rich in fat compared to animals at same age fed a grain-based diet (**D**,**F**). Depicted figures in (**A**,**B**) and (**C**–**F**) were kindly provided by Dr. Angela Schippers (Department of Pediatrics, UKA, Aachen, Germany) and Anastasia Asimakopoulou (IFMPEGKC, UKA, Aachen, Germany), respectively.

In cardiovascular research, WDs enriched in cholesterol, cholate, sucrose, and/or saturated fatty acids have also atherogenic effects and are frequently used to induce or accelerate atherosclerosis in mice [134,135].

Diets highly enriched in fructose rapidly induce an early diabetic state in mice [136,137]. In the liver fructose can be converted in several steps to glycerol-3-phosphate and metabolized by de novo lipogenesis to fatty acids, which can then be esterified to triglycerides (Figure 9A). Therefore, the chronic intake of excess dietary fructose leads to increased formation of triglycerides that accumulate in the liver (Figure 9B), insulin resistance and formation of very low density lipoprotein, attributes that are hallmarks in NAFLD [46].

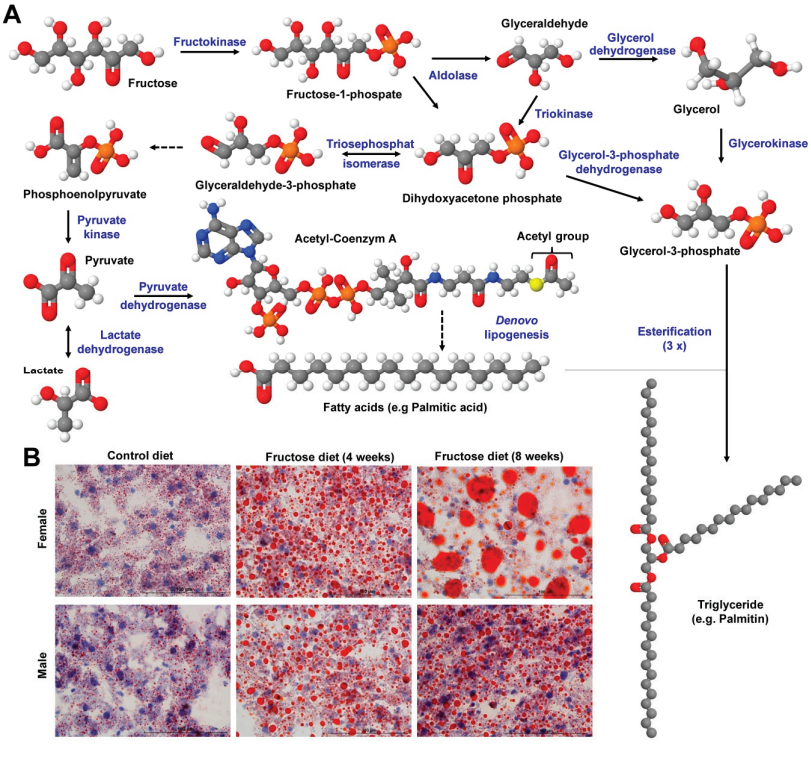

Figure 9. Fructose metabolism and consequences of increased fructose uptake. (**A**) The metabolism of fructose is initiated by phosphorylation of fructose to fructose-1-phosphate, which is subsequently hydrolyzed to form dihydroxyacetone phosphate and glyceraldehyde. Glyceraldehyde can also be converted to dihydroxyacetone phosphate or metabolized to glycerol 3-phosphate. Dihydroxyacetone phosphate can be isomerized via glycerol to glyceraldehyde 3-phosphate. Dihydroxyacetone phosphate can be further reduced to glycerol-3-phosphate or converted into glyceraldehyde 3-phosphate, and subsequently sequentially to phosphoenolpyruvate, pyruvate, and lactate. Pyruvate is central in feeding the citric acid cycle by transferring acetyl groups to coenzyme A, which is essential for the generation of fatty acids. Fatty acids can be esterified to glycerol-3-phosphate to generate triglycerides. Compound images were prepared with the Jmol program (www.jmol.org), version 14.2.15 using the compound identification (CID) nos. 5984, 65246, 751, 753, 1005, 754, 668, 754, 107735, 444493, 91435, 985, and 11147, respectively, (**B**) feeding of a fructose-enriched diet for 4–8 weeks results in progressive accumulation of hepatic fat in mice, which become evident in Oil Red O stain. Interestingly, the fat deposition is higher in female mice than in male littermates. More details about the biological effects and pathomechanism of fructose-induced fatty liver disease can be found elsewhere [46,47].

Interestingly, fructose-induced steatosis and damage induced by feeding a diet enriched in 60% fructose for four to eight weeks was more severe in female than in male mice, suggesting that respective diets provoke gender-specific differences during progression of disease [47]. Feeding of fructose in combination with fat and cholesterol for four days was already sufficient to induce hepatic triglyceride accumulation demonstrating that individual "unhealthy" compounds within a diet can be additive or synergistic [138]. Moreover, the feeding of fructose (60%) for four to eight weeks provoked impairment of olfactory epithelium, resulting in reduced olfactory behavioral capacities [139].

Other diets are characterized by the lack of essential components. In the MCD, sulfur-containing supplements are missing that cannot be synthesized de novo. When missing the essential amino acid methionine, S-adenosylmethionine (SAM or AdoMet) representing a common co-substrate involved in transmethylation, transsufuration, aminopropylation that further blunts inflammatory reactions, cannot be synthesized [140]. The lack of this compound results in lower quantities of cysteine, lecithin, phosphatidylcholine and many other macromolecules (Figure 10A) provoking significant fat accumulation and fibrosis progression in liver (Figure 10B).

Chronic shortage in methionine is, therefore, associated with a progressive physiopathology characterized by increased oxidative stress, hepatic upregulation of pro-inflammatory and pro-fibrogenic genes, liver damage as indicated by increased levels of aminotransferases, and manifestation of other NASH-associated symptoms [13]. Similarly, a shortage in choline, which is an integral part of phosphatidylcholine, sphingomyelin, and acetylcholine, results in significant intrahepatic lipid accumulation through a decreased production of very low density lipoproteins (VLDL), down-regulation of key enzymes involved in triglyceride synthesis, and impaired *de novo* lipogenesis [13]. As a consequence, harmful reactive oxygen species (ROS) are generated and the inefficient β-oxidation causes ballooning of hepatocytes, diffuse necrosis, and hepatic fibrogenesis, and on long-term liver cancer [13,141].

Cholesterol-enriched diets are widely used in studies investigating aspects of the metabolic syndrome. When mice were fed with a high (1%) cholesterol diet for 12 weeks, animals developed hyperlipidemia, hyperinsulinemia, and showed hepatocyte hypertrophy with extensive intracellular accumulation of lipid vacuoles and droplets [142]. It is suggested that in atherogenic diets, which are enriched for example in cholesterol and cholic acid, cholesterol is the key component driving oxidative stress resulting in steatohepatitis and insulin resistance [143]. In addition, these diets induced immune-related responses that may be related to liver damage in 12 inbred mouse strains tested [144].

In sum, these examples demonstrate that "unhealthy" diets enriched in or lacking of ingredients usually part of a balanced diet are suitable to provoke hepatic damage. Therefore, these diets are most popular in biomedical research to investigate mechanisms of initiation and progression of liver disease. However, many of these studies draw conclusions by comparing health aspects of animals fed a grain-based diet with a purified diet such as HFD. However, the effects of the dietary fat will be confounded with the effects of other components that differ between the diets. This fact has been already critically highlighted twelve years ago in a thought-provoking commentary in which 35 studies published in five prestigious high-impact journals were critically evaluated in regard to their performance [145] and this trend has continued as demonstrated by a more recent survey of a larger sampling of the same journals [11]. This exemplarily illustrates the fact that it is critical to draw conclusions when comparing dietary effects obtained in animals receiving either "grain-based diets" or "purified diets". Although diets are normally produced in fixed formulation, minor differences might also result when comparing findings obtained with diets produced by different companies. However, these variations should be relatively negligible.

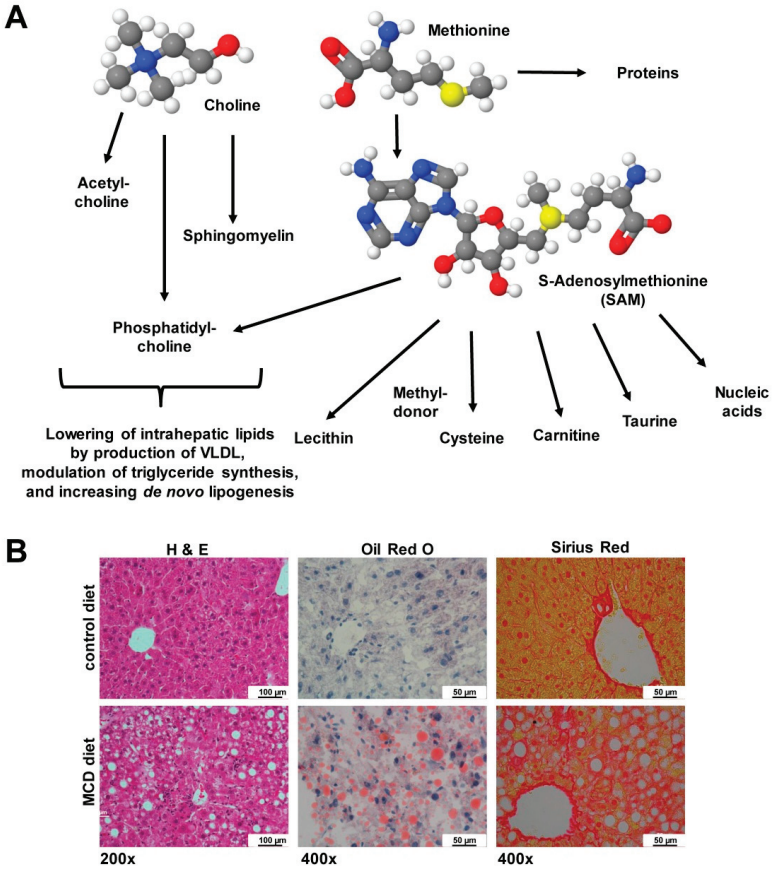

Figure 10. Choline and methionine are essential dietary supplements. (**A**) Methionine is an essential sulfur-containing amino acid that is part S-adenosylmethionine (SAM), which is indispensable as a methyl group donor in pathways driving synthesis of nucleic acids, proteins, lipids, and secondary metabolites. Choline is an integral part of phosphatidylcholines, sphingomyelins and necessary precursor for the synthesis of the neurotransmitter acetylcholine. Choline is necessary for production of very low density lipoproteins (VLDL), down-regulation of key enzymes involved in triglyceride synthesis, and proper function of de novo lipogenesis. Compound images were prepared with the Jmol program using the compound identification (CID) nos. 305, 6137, and 34755. (**B**) Mice fed a methionine-choline deficient (MCD) diet for four weeks develop severe hepatic liver damage, steatosis, ballooning, lobular inflammation, and fibrosis. In hematoxylin eosin (H & E) stain, the architectural changes are visible. In Oil Red O stain, the increased fat accumulation during the diet is assessable, while the Sirius Red stain is suitable to demonstrate increased deposition of collagens. Space bars correspond to 100 µm (H & E) or 50 µm (Oil Red O, Sirius Red). More details about the biological effects and pathomechanism of MCD diet-induced fatty liver disease can be found elsewhere [13].

9. Special Ingredients

For some studies, mouse diets are fortified with special ingredients (Figure 11). Since the mid-1990s many genetically modified mice were developed, in which the transgene is directed under the control of a tetracycline (Tet)-dependent regulatory system [146]. In these "Tet-on" or "Tet-off" systems, doxycycline is preferable as an inducer in these systems due to its high biological potency,

excellent tissue penetration, and its widespread availability [146]. This compound is rather stable in food products and its concentration is not significantly influenced by storage at room temperature or by exposure to light [146].

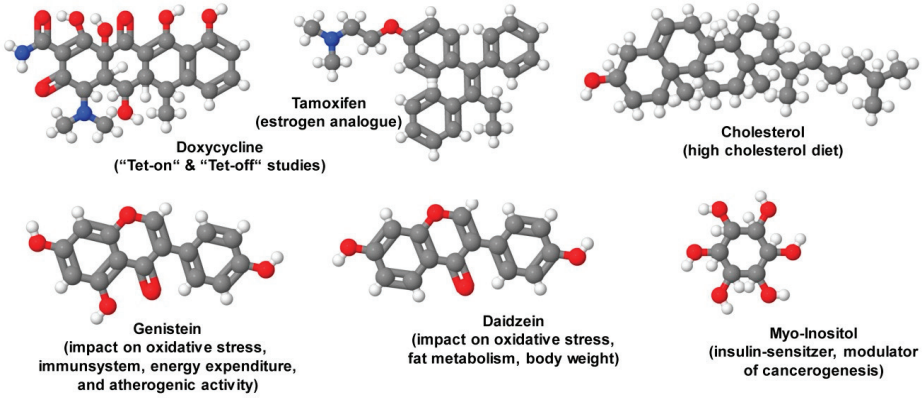

Figure 11. Some special ingredients in mouse diets used in biomedical research. Doxycycline, tamoxifen, genistein, daidzein, cholesterol, myo-inositol are compounds that are added to specific diets. Compound images were prepared with the Jmol program using the compound identification (CID) nos. 54671203, 2733526, 5280961, 5281708, 5997, and 892, respectively.

In other genetically-modified mouse systems, proteins are expressed as fusions with a modified estrogen receptor ligand binding domain. In these systems, the binding of this moiety to tamoxifen results in a conformational change that allows the fusion protein to translocate to the nucleus. The nuclear translocation of a dominant active transcription factor induces transcriptional activation of susceptible genes, while the expression of a dominant negative receptor fusion might provoke silencing of respective genes. Using this concept, also several inducible tamoxifen-dependent Cre recombinases (from English causes recombination) such as the estrogen receptor-dependent recombinase (CreER recombinases) were cloned that are widely used in biomedical research. They can direct the excision of LoxP-flanked DNA to generate genome modifications in mice [147]. However, using these models and respective diets enriched in tamoxifen, it should be noticed that this drug could influence locomotor activity, social interaction and anxiety in mice requiring critical planning of experimental design [148].

The isoflavone genistein is a phytoestrogen with antioxidant activity targeting numerous intracellular targets leading to retardation of atherogenic activity, possessing suppressive effects on both the cell-mediated and humoral components of the adaptive immune system, and inhibiting cancer progression by inducing apoptosis or inhibiting proliferation [149]. On the molecular level, it was shown that this compound inhibits a large number of enzymes, including adenosine triphosphate (ATP)-utilizing enzymes such as tyrosine-specific protein kinases, topoisomerase II and enzymes involved in phosphatidylinositol turnover [150]. Moreover, this substance has anti-angiogenic effects, modulates estrogen activity, and impacts DNA methylation and/or chromatin modification [151]. Based on this complex repertoire of activities, many mice studies have been performed with genistein-enriched diets. In one study, in which aspects of energy expenditure were analyzed in obese mice, 600 mg genistein/kg diet fed for a period of four weeks resulted in significantly increased food consumption without affecting body weight [152]. In a WD, the supplementation of 1.5 g genistein/kg diet decreased mouse food intake, body weight, and improved glucose metabolism [153]. In a study analyzing the impact of genistein on DNA methylation a concentration of 300 mg genistein/kg diet for four weeks was applied [154]. In the respective investigation, it was shown that male mice fed

a casein-based diet containing genistein showed significantly higher DNA methylation in prostate than control male mice [154].

Similar to the biological effects of genistein on energy expenditure, diets enriched with a related isoflavone daidzein-rich isoflavone aglycone extract at 0.6% of the diet was able to reduce HFD induced body weight gain via reduced hepatic production of triglycerides and subsequent reduction of adipose tissue mass [155]. It was, therefore, suggested that the beneficial effects of daidzein and genistein on food intake and body weight gain in mice are mediated by alterations within the liver X receptor (LXR) signaling pathway [153].

The sugar myo-inositol representing one of nine distinct stereoisomers of inositol is a structural component of many second messengers and lipids such as phosphatidylinositol and its derivatives. Interestingly, when chronically given, this substance has insulin-sensitizing potential in mice provoking a significant decrease in white adipose tissue [156]. Diets enriched in myo-inositol at 2.64 g myo-inositol/kg diet also showed potent reduction in the number, size, and stage of lesions in cancer-prone transgenic mice through triggering alterations in macrophage recruitment and phenotype switching [157].

These examples show that special diets have become an essential research tool in biomedical research. The companies specialized in the production of rodent diets offer limitless custom formulated diets for virtually each application. The fields of applications are numerous, including addition of special fibers, fat, or sugars to modulate the murine microbiome, incorporation of metals or environmental toxins such as microplastic to test their toxicity, feeding of "drug diets" to test the safety of compounds, and many others. The mentioned representative examples of special ingredients demonstrate the high diversity that is possible to modulate diets for a specific purpose. Custom research diets spiked with substances or lacking essential compounds are one critical puzzle piece of biomedical research that help to unravel individual risk factors contributing to disease formation. Usually these diets are produced in small quantities are formulated after consultation with the manufacturers.

10. Diet Coloring

Food colorants can be divided into three groups: (i) Naturally-derived colors used for food coloring may originate from crushed insects (e.g., carmine), saffron, turmeric, carrot, beet or their color-making ingredients, such as riboflavin and β-carotene. These can be extracted with or without intermediate of final change of identity from biological sources. The addition of these colors to foodstuffs only needs to be approved in the country in which the product is sold or manufactured. (ii) In addition, several mineral or synthetic inorganic colors such as iron oxide, titanium dioxide, chromium oxides are certified as natural food colorings, or for use in drugs, cosmetics, medical devices, or animal food. In particular iron oxides black, red and yellow are intended to be used as colorings and restore color to animal feeding stuffs at a recommended concentration between 500 and 1200 mg/kg without posing a risk to the environment [158]. Since these dyes are highly stable and excreted essentially unchanged in the faces of the animal, they are also considered as safe additives. In many cases, they can be added without requiring to be certified by regulatory bodies when applied in amounts not exceeding preset maximum concentrations. (iii) On the contrary, artificial food colors also categorized as synthetic food dyes or certified color additives are dyes produced by chemical synthesis. In the US, these synthetic compounds must be approved for their usage by the FDA. Once approved, these food dyes are typically named by Federal Food, Drug and Cosmetic Act (FD&C) numbers, while in the European Union and Switzerland certified color additives are classified with European (E) numbers. In some cases, these synthetic dyes are classified as "coal-tar colors" because they were originally produced from petroleum or coal. Actually, the usage of seven colorings and their lakes are permitted in food products in the US (Table 8, Figure 12).

Table 8. Dyes allowed for artificial coloring in the US.

Food and Drug Administration (FDA) Name	Organic Compound	Color	E Number	ADI * (mg/kg bw/d)	Chemical Abstracts Service (CAS) Number
Federal Food, Drug and Cosmetic Act (FD&C) Blue No. 1	Brilliant Blue FCF (Acid Blue 9, D&C Blue No. 4, Atracid Blue FG)	blue	E133	0–6	3844-45-9
FD&C Blue No. 2	Indigotine (Indigo Carmine, Acid Blue 74, Murabba, Sachsischblau)	indigo	E132	0–5	860-22-0
FD&C Green No. 3	Fast Green FCF (Food Green FCF, Food Green 3, Green 1724)	green	E143 ***	0–12.5	2353-45-9
FD&C Red No. 3	Erythrosine (Erythrosin B, Acid Red 51, Pyrosin B, Food Red No. 3)	pink	E127	0–0.1	16423-68-0
FD&C Red No. 40	Allura Red AC (Food Red 17, Curry Red)	red	E129	0–7	25956-17-6
FD&C Yellow No. 5	Tartrazine (Tatrazol Yellow, Acid Yellow 23, Food Yellow 4)	yellow	E102	0–10	1934-21-0
FD&C Yellow No. 6	Sunset Yellow FCF (Orange Yellow S, Food Yellow 3)	orange	E110	0–4	2783-94-0
Citrus Red 2 **	Citrus Red (2,5-Dimethoxy-1-Phenylazo-2-naphthol, CI 12156, CI Solvent Red 80)	red	E121 ***	Only approved for use to color orange peels (US)	6358-53-8
Orange B **	Acid Orange 137 (LS-128771, Schembl132534)	orange	not allowed ***	Only approved for use in hot dog and sausage casings (US)	53060-70-1

* Acceptable daily intake (ADI) values are given for humans and were taken from the Joint FAO/WHO Expert Committee on Food Additives (JECFA, https://www.who.int/foodsafety/areas_work/chemical-risks/jecfa/en/) or from the Internationally Peer Reviewed Chemical Safety Information (http://www.inchem.org). Abbreviations used are: bw, body weight; ** Dye allowed by the FDA for limited applications. *** The usage of this dye in food products is forbidden in the EU.

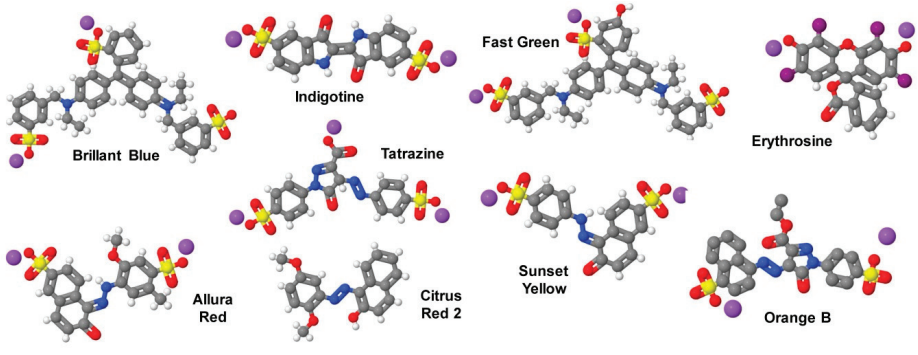

Figure 12. Artificial coloring of mouse diets. The artificial dyes Brilliant Blue, Indigotine, Fast Green, Erythrosine, Allura Red, Tatrazine, Citrus Red 2, Sunset Yellow, and Orange B or using their lakes has been permitted by the US Food and Drug Administration to color food products. These dyes are also approved for mouse diets. Compound images were prepared with the Jmol program using the compound identification (CID) nos. 19700, 2723854, 16887, 12961638, 33258, 164825, 22830, 17730, and 11685735, respectively.

Usually the dyes used in rodent diets are applied as ionic salts rendered partially insoluble by interaction with a metal such as calcium or aluminum. The FDA defines these water-soluble dyes as "lakes", in which the proportions of dye to metal are more or less fixed and given in percentages (e.g., FD&C Red No. 40, aluminum lake, 36–42%).

Commonly, the dyes for coloring of food are used to modify the appeal of food for humans [159]. However, in comparison to humans, mice do not have the visual ability to distinguish the abundance

of colors [160]. Instead, the uptake of food by a mouse is strongly dependent on smell perception and olfactory system that are extremely important for controlling energy homeostasis [161]. The coloring of mouse diets is, therefore, in principle of no importance for the animals. However, the coloring has some useful properties. They allow the researcher to distinguish one diet from another and they ensure that contamination and transmissions to other diet products during the production pipelines can be recognized. In the following, we will give some short information about the artificial colors used for food coloring.

Brilliant blue (FD&C Blue No. 1, E133) is a reddish-blue triarylmethane water-soluble dye used as a blue colorant. It has reasonable stability when exposed to light, heat and acidic conditions, but it has overall low oxidative stability [162]. This dye is considered harmless and most of the dye is excreted undigested [163]. There is no evidence that this dye in rats or mice is carcinogenic, or neurotoxic [164]. However, this dye can act as purinergic inhibitor without pharmacological selectivity, thereby modulating some organ and tissue functions [162]. In addition, a recent report has demonstrated that this dye showed significant greater absorption in septic patients with reduced intestinal barrier function [165]. The daily maximum FDA-approved uptake of Brilliant blue for humans is 12.5 mg/kg body weight/day, while the EU scientific committee suggested an acceptable daily intake (ADI) of 10 mg/kg body weight/day [162].

Indigotine (FD&C Blue No. 2, E132) or indigo is a dark blue water-insoluble anionic pyrrole-based dye originally isolated from the leaves of certain tropical plants. Nowadays this dye is one of the most used coloring agents in the textile industry and synthesized by various methods [166]. In toxicity studies, indigo carmine, representing an organic water-soluble salt derived from indigo by sulflonation, showed no genotoxicity, developmental toxicity or modification of hematological parameters. An ADI up to 5 mg/kg body weight is presently considered as harmless [167]. Moreover, in traditional Chinese medicine, indigo as "Qing-Dai" alone or in combination with other compounds is used as an overall safe and effective drug for treatment of sun stroke, convulsions associated with epilepsy, cough, chest pain, hemoptysis, and phlegm and childrens convulsions [167]. In rodents, the majority of this dye is not absorbed, but readily broken down in the gastrointestinal tract to 5-sulfoanthranilic acid that is absorbed and excreted mostly in the urine [164].

Fast Green FCF (FD&C Green No. 3, E143) is a FDA-approved triphenylmethane dye, while its usage as a food dye is prohibited in the EU [168]. When administered orally 200 mg of this dye to rats, the dye was excreted unchanged in the faeces and no dye was found in the urine [169]. Mice fed diets containing up to 2% Fast Green FCF for 78 weeks, showed no lesions attributed to feeding of the color [170]. The estimate of temporary ADI for man is set up to 12.5 mg/kg body weight [171].

Erythrosine (FD&C Red No. 3, E127) is a cherry-pink poly-iodinated xanthene used as artificial red colorant in foods, drugs and cosmetics [172]. In the past, this dye was commonly used in many countries but is less commonly used in the US, where it is most often replaced by Allura Red AC (FD&C Red No. 40). Chronic toxicity and carcinogenicity studies performed in rats and mice revealed an increased incidence of thyroid follicular cell hyperplasia and adenomas in animals that received 4% erythrosine in the diet for 30 months following in utnero exposure [173]. However, this dye is non-mutagenic and thus the observed tumorigenic activity is most likely not the result of genotoxic initiation [174]. However, the dye has negative effects on thyroid function and therefore the temporary ADI is only in the range of 0–0.05 mg/kg body weight [175].

Allura Red AC (FD&C Red No. 40, E129) is a highly popular red azo dye that may cause allergic reaction such as urticaria or asthma, especially when administered together with orther synthetic color additives [167]. However, in general this dye at 0–7 mg/kg of body weight per day is considered as safe [167]. In the US population, Allura Red AC belongs to the three highest cumulative eaters-only exposures of FD&C color additives in food products [176,177]. Although the European food safety authority expressed concerns about the usage of Allura Red AC as a food color additive, the dye has no genotoxic activity in different test systems [178].

Tartrazine (FD&C Yellow No. 5, E102) is a water-soluble yellow monoazo dye used all over the world for food coloring. In a community-based, double-blinded, placebo-controlled food study, this dye in a mix with other artificial color additives provoked increased hyperactivity in young children [179]. However, the compound has an overall low toxicity with and LD_{50} value of greater than 2 g/kg body weight and an ADI for humans of 0–7.5 mg/kg body weight was established [177]. Similarly, in mice high-dose level in excess of this ADI were shown to produce only a few adverse effects in neurobehavioural parameters during the lactation period that were however unlikely produce any adverse effects in humans [180].

Sunset Yellow FCF (FD&C Yellow No. 6, E110) is an orange azo dye supposed to have no carcinogenicity, genotoxicity, or developmental toxicity in mice [181]. According to the WHO/FAO guidelines, the ADI was increased in year 2014 from 0–1 mg/kg body weight per day to 0–4 mg/kg body weight [182]. When high content of Sunset Yellow FCF (up to 5% for 23 months) were fed to mice, the mortality rate was not significantly different than in mice receiving no dye and the histopathological changes in organ and tissue observed were considered unrelated to the dietary administration of Sunset Yellow FCF [182].

Citrus Red (Citrus Red 2, E121) is an yellow to orange dye. Testings in mice showed that the feeding of diets containing 3% Citurs Red 2 caused increased morbidity and mortality in both sexes [183]. Based on a number of similar reports suggesting that Citrus Red 2 has carcinogenic effects, the FDA approved this dye only for limited applications such as coloring the peel of oranges, while in the EU it is not permitted at all [168]. However, there it was recommended that this dye should not be used as a food additive [184]. Therefore, this dye should not be incorporated into rodent diets.

Similarly, Acid Orange 137 (Orange B) is approved in the US for use in small traces (150 ppm) only in Frankfurter and sausage casings, while it is forbidden as a food additive in the EU [164,184]. Structurally, it is a pyrazolone dye that is reduced in the gut to form naphthionic acid [184]. In rodents this dye induces lymphoid atrophy of the spleen, bile-duct proliferation, and moderate chronic nephritis when applied for long-term [184]. Therefore, the usage of this dye as a food additive is forbidden in the EU [168].

In sum, the FDA has approved seven synthetic color additives for usage in food products. Three of them (Fast Green FCF, Citrus Red No. 2, and Orange B) are not permitted in the EU as food additives. Although the impact of artificial dyes on mice has not been intensively investigated, there are some studies showing that individual artificial dyes or combinations thereof might be neurotoxic when applied in high concentration [185]. However, typically the concentration necessary to induce adverse effects in male and female mice are extremely high. Brilliant blue FCF for examples showed no adverse effects even at high dietary concentration (7354 mg/kg/day and 8966 mg/kg/day) in male and female mice for 104 weeks [186]. These concentrations are far beyond the concentrations that are used for diet coloring of mice research diets.

11. Diversity of Diet Ingredients may Confound Data Interpretation

As discussed above, many papers using nutritional models in mice draw conclusions about dietary effects from comparison of grain-based diets with purified diets [145]. However, such mismatched diets potentially hamper the investigator's ability to draw useful conclusions from otherwise well-designed studies [11]. The reproducibility of research findings is adversely affected by the use of improper control diets in metabolic disease research and the lack of adequate diet descriptions in resulting publications [11]. In many publications, grain-based diets referred vaguely as "chow diet", "normal diet" or "control diet" are compared with purified ingredient diets also named as purified diets or semi-purified diets. Grain-based diets are made with grain, cereal ingredients, and animal by-products that may be somewhat variable from formulation to formulation, while purified diets are composed of highly refined ingredients [11]. Given these inherent differences between these diets, data produced from them should not be compared to each other or matched to one factor specifically different (e.g., fat or sugar composition). In particular, soluble fibers fermented

by bacteria in the gut to SCFAs can for example change the gut pH, absorption of bile acids, and chelation of minerals [11]. Exemplarily, Chassaing and coworkers have demonstrated impressively that mismatched diets can result to erroneous conclusions [187]. In their study, the authors investigated the extent to which HFD-induced adiposity is driven by fat content vs. other factors that differentiate purified HFD, grain-based diet, and compositionally-defined diets (i.e., purified diets). Interestingly, the study revealed that high-fat content and lack of soluble fiber are both acting as obesogenic factors promoting rapid and marked loss of cecal and colonic mass and increased adiposity [187]. Therefore, the diet with its ingredients has to be considered as a key environmental factor that critically affects the outcome of a specific experiment. Properly matched "control diets" are therefore an indispensable prerequisite to draw conclusions regarding diet-driven phenotypic differences in respective studies.

12. Conclusions

Nutritional factors are crucial in laboratory animal science. To guarantee reproducibility of mouse experiments, it is necessary that they receive reliable food with constant composition. There are many providers that have concentrated on the production of grain-based and purified diets. Some are certified and produce their products according to national and international guidelines. The ingredients used are analyzed extensively and the production process guarantees nutrient stability and purity. Besides modification of the different ingredients of a diet, diets can be produced in varying shape, grains and colors using approved non-toxic food dyes. γ-rays and pasteurization are frequently used to sterilize diets fed in SPF facilities. However, these treatments might result in vitamin loss and formation of toxic substances, including acrylamide and peroxide radicals. An important but largely underestimated problem in conducting animal experimentation relying on nutritional models is the impact of confounding factors when choosing unsuitable control diets. These factors might impact the outcome of a specific experiment and incorrect conclusions. Confounding factors in this context are all ingredients differing between the control diet and the intervention diet. Likewise, the coloring with dyes such as Erythrosine and Tartrazine that have already shown to have biological effects in mice or humans should be ommitted for diet coloring. If an investigator has special requirements or wishes for his dietary interventions, it is urgently advisable to contact the manufacturer of the diet product before starting an animal experiment. In most cases, the manufacturers of diets offer consultations with expert nutritionists to assist the scientists in the selection of the right diet for the planned study requirement.

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

Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ADI	Acceptable daily intake
AIN	American Institute of Nutrition
ANSES	Agency for Food, Environmental and Occupational Health & Safety (L'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail)
BARQA	British Association for Research Quality Assurance
CEN	European Committee for Standardization
CID	Compound identification

DHAc	Docosyhexaenoic acid
DIN	German Institute for Standardization (Deutsches Institut für Normierung)
DIO	Diet-induced obesity
EPA	Environmental Protection Agency
EPAc	Eicosapentaenoic acid
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FD&C	Federal Food, Drug and Cosmetic act
FDA	Food and Drug Administration
FFC	fat, fructose and cholesterol-rich diet
FSA	Food Standards Agency
GLP	Good laboratory practice
GMP	Good manufacturing practices
HDL	High density lipoprotein
HFD	High-fat diet
ISO	International Organization for Standardization
JECFA	Joint (FAO/WHO) Expert Committee for Food Additives
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
MCD	Methionine-choline-deficient diet
MND	Menadione nicotinamide bisulfite
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NRC	National Research Council
SAM	S-Adenosylmethionine
SPF	Specified pathogen free
SOP	Standard operating protocol(s)
WD	Western diet
WHO	World Health Organization

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nutrients

MDPI

Review

Dietary Strategies for Metabolic Syndrome: A Comprehensive Review

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Abstract: Metabolic syndrome is a cluster of metabolic risk factors, characterized by abdominal obesity, dyslipidemia, low levels of high-density lipoprotein cholesterol (HDL-c), hypertension, and insulin resistance. Lifestyle modifications, especially dietary habits, are the main therapeutic strategy for the treatment and management of metabolic syndrome, but the most effective dietary pattern for its management has not been established. Specific dietary modifications, such as improving the quality of the foods or changing macronutrient distribution, showed beneficial effects on metabolic syndrome conditions and individual parameters. On comparing low-fat and restricted diets, the scientific evidence supports the use of the Mediterranean Dietary Approaches to Stop Hypertension (DASH) diet intervention as the new paradigm for metabolic syndrome prevention and treatment. The nutritional distribution and quality of these healthy diets allows health professionals to provide easy-to-follow dietary advice without the need for restricted diets. Nonetheless, energy-restricted dietary patterns and improvements in physical activity are crucial to improve the metabolic disturbances observed in metabolic syndrome patients.

Keywords: metabolic syndrome; dietary pattern; Mediterranean diet; plant-based diet; DASH diet; low-carbohydrate diet; high-protein diet; low-fat diet

1. Introduction

Following unhealthy dietary patterns and sedentary lifestyles has led to a notable increase in the prevalence of overweight and obesity worldwide. Non-communicable chronic diseases (NCDs) related to unhealthy dietary patterns and weight gain have expanded in parallel, being the major cause of morbidity and mortality both in developed and underdeveloped countries [1]. Among NCDs, cardiovascular diseases (CVD) and type 2 diabetes mellitus (T2DM) are public health priorities, not only for their high prevalence and outcomes but also for the huge economic burden imposed on the health system [2,3].

Metabolic syndrome (MetS) is a clinical condition characterized by a clustering of metabolic risk factors, which is defined by the simultaneous occurrence of at least three of the following components: central obesity, dyslipidemia, impaired glucose metabolism, elevated blood pressure (BP), and low levels of high-density lipoprotein cholesterol (HDL-c), according to the consensual definition of the International Diabetes Federation, the American Heart Association, and the National Heart, Lung and Blood Institute [4]. In developed countries, the prevalence of MetS has risen up to 20–25% in the adult

population, and its incidence continues to increase over time [5–8]. In Spain, the prevalence of MetS is currently reaching epidemic proportions, affecting approximately 22.7% of the population, taking into account that its incidence increases with age [5]. In addition, MetS increases the risk of T2DM onset and major cardiovascular events by two-fold and five-fold, respectively, and other chronic disease such as cancer, neurodegenerative diseases, non-alcoholic fatty liver disease, the risk of reproductive, lipid and circulatory disorders, atherosclerosis, and all cause-mortality are also increased [8–13].

Recent evidence has demonstrated the association between the incidence and prevention of MetS and modifiable lifestyle factors, especially dietary habits. Steckhan et al. analyzed the positive effects of different dietary approaches on MetS inflammatory markers [14]. Regarding the prevention of MetS, Godos et al. also conducted a meta-analysis to demonstrate the preventive role of the promotion of healthy dietary patterns to reduce the prevalence of MetS [15]. Furthermore, some sub-studies from the PREDIMED-Plus cohort showed associations between some dietary components of the traditional Mediterranean Diet (MedDiet) and improvement in MetS components [16–20]. The aim of the present review was to analyze the potential benefits of different dietary approaches on MetS status and their use as efficient strategies to prevent and treat MetS and its comorbidities.

Dietary Patterns

A single-nutrient dietary intervention has several limitations, and dietary advice must be focused on the overall dietary pattern as part of MetS treatment. Recent evidence supports the implementation of healthy food-based dietary interventions instead of calorie or isolated nutrient restriction [1,21] diets. The health benefits, regarding MetS, of dietary macronutrient patterns and different dietary approaches are summarized in Table 1.

Table 1. Dietary strategies and potential health benefits for Metabolic Syndrome (MetS).

Dietary Pattern	Nutritional Distribution	Improvements in MetS Criteria	Ref.
Mediterranean diet	▪ 35–45% kcal/d from total fat (mainly MUFA [1], EVOO and nuts being the principal source) ▪ 35–45% kcal/d from CH ▪ 15–18% kcal/d from protein	Reduction of CVD incidence and outcomes Decreased BP (systolic and diastolic) Inverse association with mortality Improvements in dyslipemia Decreased incidence of T2DM	[22–29] [15,26] [24,30] [26] [12,22,23,29,31]
DASH diet	▪ Total fats 27% kcal/d ▪ Saturated fats 6% kcal/d ▪ Dietary cholesterol ▪ CH 55% kcal/d ▪ Proteins 18% kcal/d	Reduction of BP (systolic and diastolic) Reduction in BMI and waist circumference Improvement in cardiometabolic profile Reduction in T2DM incidence	[32,33] [34,35] [36–39] [40]
Plant-based diets	▪ Reduction or restriction of animal-derived foods ▪ High intake of plant-source foods ▪ Fat profile rich in UFAs	Reduction of BP (systolic and diastolic) Decreased body weight and risk of obesity Reduction of the risk of CVD Decreased all-cause mortality Decreased risk of T2DM	[41,42] [43–45] [46] [43,47,48] [43,47,48]
Low CH diets and very low CH diets (ketogenic diets)	▪ <50% kcal/d from carbohydrates and <10% kcal/d from CH in ketogenic diets ▪ High protein (20–30% kcal/d) ▪ High fat intake (30–70% kcal/d)	Weight-loss and weight-loss maintenance Reduction of DBP Reduction of LDL-c and triglycerides levels Increase of HDL-c levels Improvements in insulin resistance Reduction of HbA1c levels	[49–52] [52] [49–51] [49–51] [53,54] [49,51]
Low-fat diet	▪ <30% kcal/d from total fat (<10% of saturated fat) ▪ 15–17% kcal/d from protein ▪ 50–60% kcal/d from CH	Reduction of BP (systolic and diastolic) Short-term improvement of cholesterol profile Short-term weight loss Reduced risk of all-cause mortality	[33,55] [33,55] [55] [56]
High protein diet	▪ High protein (20–30% kcal/d) or 1.34–1.50 g/Kg body weight/d from protein ▪ Low CH (40–50% kcal/d)	Reduction of triglycerides levels	[57,58]

Table 1. *Cont.*

Dietary Pattern		Nutritional Distribution	Improvements in MetS Criteria	Ref.
Other dietary patterns and strategies	Nordic diet	▪ High content of whole-grain high-fibre products ▪ Low in meat and processed foods	Reduction of BP (systolic and diastolic) Increase of HDL-c levels	[59] [59]
	Intermittent fasting	▪ Fasting for a long period of time	Weight loss Improvements in insulin resistance Improvements in dyslipidaemia Reduction of BP (systolic and diastolic) Decreased risk of T2DM Decreased risk of CVD	[60–62] [60–62] [60–62] [60–62] [63] [63]

[1] Monounsaturated fatty acids, MUFA; extra virgin olive oil, EVOO; carbohydrates, CH; cardiovascular disease, CVD; blood pressure, BP; type 2 diabetes mellitus, T2DM; Dietary Approaches to Stop Hypertension, DASH; unsaturated fatty acids, UFAs; body mass index, BMI; diastolic blood pressure, DBP; low-density lipoprotein cholesterol, LDL-c; high-density lipoprotein cholesterol, HDL-c, glycated hemoglobin, HbA1c; monounsaturated fatty acids, MUFA.

2. Mediterranean Diet

The MedDiet refers to the dietary pattern, culture and culinary techniques adhering to countries and populations living in the Mediterranean Sea basin [64]. This dietary pattern has stimulated a great deal of scientific evidence, demonstrating the potential health benefits associated with adherence, and the primary and secondary prevention of many health outcomes, such as CVD, T2DM, and MetS [10,22]. Recent scientific evidence concluded that the MedDiet not only has beneficial effects on health but also has beneficial effects on sustainability and culture [22,65]. Additionally, the MedDiet has been recognized by UNESCO as an Intangible Cultural Heritage of Humanity [66] and the 2015–2020 American Dietary guidelines referred to the MedDiet as an example of a healthy dietary pattern [21]. The MedDiet is a plant-based diet characterized by a high intake of vegetables including leafy green vegetables, fruits, whole-grain cereals, pulses, legumes, nuts, and extra virgin (cold pressed) olive oil (EVOO) as the main source of fat. Moreover, classical recipes are seasoned with sauces such as *sofrito*, whose main ingredients are olive oil, tomato, garlic, onion or leek, rich in phenolic compounds and carotenoids, such as naringenin, hydroxy-tyrosyl, lycopene and β-carotene [67]. Moderate alcohol intake of fermented alcoholic beverages such as red wine, mainly during meals, is also characteristic of the MedDiet, which also comprises a low to moderate intake of fish and poultry, and low consumption of red meat, butter, sweets, pastries and soft drinks [12,23,68]

The traditional MedDiet is a high fat and low-carbohydrate (CH) dietary pattern, which provides a 35–45% of total daily energy intake from fat, about 15% from protein, and 40–45% energy from CH [12,68]. However, the profile of this fat is mainly one of monounsaturated (MU) and polyunsaturated (PU) fatty acids (FA) and the main food sources of total fat intake are EVOO and nuts. EVOO is one of the key foods of the MedDiet and is the main contributor of monounsaturated fatty acids (MUFAs) in MedDiet countries. Oleic acid is the major component of EVOO and many studies have linked MUFA intake to improvements in insulin resistance, one of the main risk factors for MetS, and in blood lipid profile, and a reduction in both systolic and diastolic BP levels [12,24,69]. EVOO is also rich in polyphenols, which present anti-inflammatory and antioxidant effects and contribute to improving the lipid profile and endothelial function [70] Besides the beneficial effects of unsaturated fats, the whole dietary pattern characterized by the high intake of fruits and vegetables together with moderate red wine consumption provides wide nutritional components, such as antioxidant vitamins (vitamin C, E and β-carotene), phytochemicals (such as polyphenols), folates and minerals, which may exert beneficial effects [31,70].

Considering the effects of the MedDiet on MetS, Di Daniele et al. conducted a review addressing the impact of MedDiet adherence on MetS criteria, obesity and adipose tissue dysfunction [10]. The authors reported that prescription of the MedDiet can be used as a possible therapy for MetS, as it prevents the excess of adiposity and obesity-related inflammatory response. Franquesa et al. concluded that there is a strong evidence for the effect of the MedDiet on obesity and on MetS prevention in healthy or high-CVD risk individuals, as well as on the risk of mortality in overweight or obese individuals [22]. As previously cited, a meta-analysis of 12 cross-sectional and prospective cohorts showed that higher adherence to the MedDiet was associated with a 19% lower risk of developing MetS (relative risk (RR): 0.81 (95% confidence interval (CI) 0.71 to 0.92)), and individual components, such as waist circumference and BP, were also improved (RR: 0.82 (95% CI 0.70 to 0.96); RR: 0.87 (95% CI 0.77 to 0.97), respectively) [15]. Several prospective studies observed the same protective effects in Mediterranean and non-Mediterranean countries [25–27]. The CARDIA (Coronary Artery Risk Development in Young Adults) study is a prospective study including 4713 individuals which evaluated the evolution of CVD risk factors in black and white populations in the United States [28]. They observed a lower incidence of MetS in individuals with a higher adherence to the MedDiet (Hazard ratio (HR): 0.67 (95% CI 0.49 to 0.90)) compared to those with lower adherence, showing a linear trend according to the five score categories (p for trend = 0.005) [25]. Kesse-Guyot et al. conducted a prospective 6-year follow-up with 3232 subjects in the SU.VI.MAX study to evaluate the association between different MedDiet adherence scores and the incidence of MetS. They found that participants with higher adherence had a 53% lower

risk compared to the lowest tertile of the MedDiet score (odds ratio (OR): 0.47 (95% CI 0.32 to 0.69) and 0.50 (95% CI 0.32 to 0.77 for each MedDiet score) [26]. In addition, MedDiet adherence scores were associated with improvements in some individual criteria for MetS, such as waist-circumference, BP, triglycerides and HDL-c levels [26]. Moreover, lower MetS prevalence was observed in Korean adults with medium to high MedDiet adherence (OR: 0.73 (95% CI 0.56 to 0.96) and 0.64 (95% CI 0.46 to 0.89), respectively) [30].

MedDiet adherence has been inversely associated with the incidence of CVD and mortality, as well as cancer and degenerative diseases [23,71]. In the case of CVD, the MedDiet is associated with clinically meaningful reductions in the risk of developing the main CVD outcomes, including coronary heart disease and stroke [72]. In a prospective cohort study with 25,994 healthy women from the US Women's Health Study, Ahmad et al. observed an inverse association between the highest MedDiet adherence score and the incidence of CVD compared to the lowest score (HR: 0.72 (95% CI 0.61 to 0.86), p for trend < 0.001) [73]. Among the health effects observed, MedDiet interventions have shown improvements in body composition by reducing total and segmental fat, which might have an effect on metabolic profile [10]. Furthermore, the MedDiet has contributed to a decrease in the incidence of T2DM and CVD, while lessening severity and associated complications in individuals who have already been diagnosed [12,22,23,29,31]. Due to the health benefits associated with this easy-to follow dietary pattern, the MedDiet should be considered as one of the first treatment strategies for the prevention and management of MetS.

3. DASH Diet

In 1997, the Dietary Approaches to Stop Hypertension (DASH) diet became a promising strategy for the treatment of high BP [74], and subsequent randomized clinical trials (RCTs) have supported this evidence [32]. This eating pattern promotes vegetables, fruits, whole grains, low- or free-fat dairy products, legumes and nuts intake, while restricting the intake of red and processed meat and sugar-sweetened beverages [74,75]. The DASH diet is characterized by a low-fat content (27% of daily calorie intake from fat), especially saturated fats (6% of energy) and dietary cholesterol (150 mg/d approximately), and reduced sodium content (from 1500 to 2300 mg/day), but it is rich in fiber (>30 g/day), potassium, magnesium and calcium compared to other dietary patterns [55,76]. The DASH diet has proven to be a useful strategy for the treatment of hypertension [32,33,55,77], and several epidemiological studies have associated higher adherence to the DASH diet with a better cardiometabolic profile [34,36–39,78–80]. In a meta-analysis of several cohort studies, Schwingshackl et al. reported that higher adherence to the DASH diet was associated with a significant reduction in the risk of all-cause mortality (RR: 0.78 (95% CI 0.77 to 0.80), the incidence of or mortality by CVD and cancer (RR: 0.78 (95% CI 0.76 to 0.80); RR: 0.84 (95% CI 0.82 to 0.87), respectively) and the incidence of T2DM (RR: 0.82 (95% CI 0.78 to 0.85)) [40].

Regarding the use of DASH diet as an approach for the treatment of hypertension, a recent meta-analysis of 30 RCT with 5545 hypertensive and non-hypertensive participants concluded that the DASH diet together with lifestyle interventions significantly decreased systolic and diastolic BP measurements compared with a control diet (mean differences: −3.2 mm Hg (95% CI −4.2 to −2.3) and −2.5 mm Hg (95% CI −3.5 to −1.5), respectively) [32]. This effect was more pronounced when sodium intake was lower than 2400 mg/d, in subjects under the age of 50, and in participants with hypertension but without antihypertensive medication [32]. Moreover, on comparing the antihypertensive effects of the DASH diet with 13 other eating patterns (including low-fat diet, Nordic diet, MedDiet, Paleolithic diet and low-sodium diet), the DASH diet was the most effective, especially in comparison with low-fat diets [33]. In contrast, Ge et al. identified the Paleolithic and Atkins diets as the most effective dietary patterns for both systolic and diastolic BP management after six months of intervention compared to usual dietary advice, although this effect was not observed after one year of intervention [55].

The DASH diet intervention has also shown potential effects against excess body weight and abdominal obesity [35]. Middle-term dietary interventions have shown a significant reduction in

body mass index (BMI) (weighted mean difference: −0.42 kg/m2 (95% CI −0.64 to −0.20)) and waist circumference (−1.05 cm (95% CI −1.61 to −0.49)) [35]. Nevertheless, in overweight or obese individuals, DASH dietary approaches showed significant weight loss compared to other dietary patterns (−3.63 kg (95% Credible Interval −2.52 to −4.76)) whereas this weight loss was lower after one year of intervention (−3.08 kg (95% Credible Interval −0.48 to −5.66)) [55].

The results are not consistent in the case of blood lipoproteins [55,77]. Ge et al. did not observe significant differences in HDL-c or low-density lipoprotein-cholesterol (LDL-c) levels after a DASH dietary intervention versus usual diet [55], whereas in a meta-analysis of 1917 participants with some CVD risk factors, Siervo et al. observed a reduction in total cholesterol and LDL-c levels after the DASH intervention (mean differences: −0.20 mmol/L (95% CI −0.31 to −0.10) and −0.10 mmol/L (95% CI −0.20 to 0.01), respectively), but reported no significant differences in HDL-c and triglyceride levels [77]. Similar results were obtained in a recently published controlled trial in 80 T2DM patients after 12 weeks following the DASH diet compared to an antidiabetic diet based on American Diabetes Association guidelines [81]. Both dietary interventions significantly reduced triglycerides, total cholesterol and very-low-density lipoproteins.

Epidemiological evidence suggests an association between higher adherence to the DASH diet and a better cardiometabolic profile and lower risk of CVD [36–39]. A cross-sectional study of 1493 adults showed that higher adherence to the DASH diet was associated with 48% less risk of developing MetS, whereas BMI, waist circumference, pro-inflammatory markers and adiposity measures were significantly lower compared to individuals with lower adherence [34]. Interestingly, Ashari et al. observed that higher adherence to the DASH diet was associated with a 64% lower risk of MetS in 425 healthy children and adolescents from 6–18 years of age [82]. In addition, the authors also observed inverse associations among adherence to the DASH diet and BP, fasting plasma glucose levels and abdominal obesity [82]. In this sense, adaptation of the DASH diet to type 1 diabetes glucose requirements (a reduction in CH of around 10% and 15% increase in fat content) resulted in better glucose control and improved the quality of the whole diet, showing a higher intake of fruits, vegetables, fiber and protein compared to the usual intake [83].

The health benefits associated with the DASH diet are probably due to its nutritional quality and distribution. The DASH diet is rich in vegetables and fruits, which translate into high potassium, magnesium and fiber intake, and these nutrients have shown to have a role in BP control, glucose metabolism and insulin response [84]. Furthermore, vegetables and fruits are the main food source of antioxidants and polyphenols, which have been linked to better glucose and insulin blood levels [84]. Moreover, it is limited in sodium and fat, mainly saturated fatty acids (SFA), which are closely related to CVD [84]. Nonetheless, Pickering et al. suggested that the potential health effects of the DASH diet are dependent on eating pattern adherence, with subjects with lower adherence to the DASH diet showing greater benefit from DASH dietary interventions in BP control than those with higher adherence before the dietary intervention [85]. Nonetheless, the commitment and implication of the patient are critical in all life-style interventions based on dietary modifications [86,87].

4. Plant-Based Diets

Plant-based diets include a wide variety of dietary patterns, which are characterized by a reduction or restriction in animal-derived food intake and the promotion of plant-source food intake, such as fruits, vegetables, nuts, legumes, and grains. Among plant-based diets, strict vegetarian diets, also known as vegan diets, are defined by the exclusion of all animal-derived products, including dairy products, eggs and honey; lacto-vegetarian diets restrict animal food intake except for dairy products; lacto-ovo-vegetarian diets exclude meat, seafood and poultry but include eggs and dairy products; and pesco-vegetarians or pescatarians are similar to lacto-ovo-vegetarian but include fish [88]. Despite the fact that plant-based diets are defined by the exclusion of some or all animal products, recent evidence defines plant-based diets as dietary patterns that promote a reduction in animal-source food intake along with an increase in plant-based food intake, such as the MedDiet [41,88–90].

Plant-based diets have consistently been associated with beneficial cardiometabolic effects, specifically with a lower risk of developing MetS and all of its components [91]. Moreover, these dietary patterns are associated with decreased all-cause mortality and a decreased risk of obesity, T2DM and CVD [43,47,48]. Some studies have found a lower risk of mortality from ischemic heart disease in vegetarians compared with non-vegetarians [43]. Additionally, recent systematic reviews and meta-analyses found significant associations between adherence to the MedDiet and DASH diets and a 38% and 20% lower risk of CVD, respectively, while a 28% reduction in the risk of coronary heart disease was observed following a vegetarian diet [46].

Regarding BP, a meta-analysis of seven RCTs reported a mean reduction of 4.8 mmHg in systolic BP (95% CI −3.1 to −6.6; $p < 0.001$) and a 2.2 mmHg reduction in diastolic BP (95% CI −1.0 to −3.5; $p < 0.001$) in participants following a vegetarian diet compared to an omnivorous diet [42]. These results were confirmed by the same authors in a meta-analysis of 32 observational studies including 604 participants, in which an association was observed between vegetarian diets and reductions in systolic and diastolic BP (−6.9 mmHg (95% CI −9.1 to −4.7; $p < 0.01$) and −4.7 mmHg (95% CI −6.3 to −3.1; $p < 0.01$), respectively) [41,42].

The effects of plant-based diets on blood lipid concentrations are controversial. Wang et al. conducted a meta-analysis of 11 RCTs to evaluate the effects of vegetarian diet on triglycerides, LDL-c, HDL-c and non-HDL-c levels [92]. Total cholesterol levels, LDL-c and HDL-c, were significantly reduced after following a vegetarian diet compared to an omnivorous control diet (0.36 mmol/L (95% CI 0.55 to 0.17; $p < 0.001$), 0.34 mmol/L (95% CI 0.57 to 0.11; $p < 0.001$) and 0.10 mmol/L (95% CI 0.14 to 0.06; $p < 0.001$), respectively). No significant effects were observed for triglyceride levels. This study also described a significant weight-loss in participants who followed the vegetarian compared to the omnivorous diet (−2.88 kg (95% CI −3.56 to −2.20; $p < 0.001$)). Similar results were observed in another meta-analysis of 12 RCTs involving 1151 individuals, in which subjects randomized to the vegetarian diet intervention group showed significant weight loss compared to the non-vegetarian group (mean difference −2.02 kg (95% CI −2.80 to −1.23; $p < 0.001$)) [44]. Other studies assessing plant-based dietary patterns, such as the MedDiet, have also described positive effects on body weight and waist circumference [45].

The health benefits observed are mainly explained by the nutritional quality of plant-based diets as they promote the intake of a wide variety of plant-based foods while cutting down the intake of animal-derived products, such as red and processed meat, which have been associated with a higher risk of developing T2DM, CVD and certain types of cancer [93]. However, it is important to consider that the term "plant-based" does not necessary mean "healthy", as there is evidence supporting adverse health effects of the excessive intake of some plant-derived foods, such as refined grains, snacks, pastries or sugar-sweetened beverages [41,88,94]. A healthy plant-based diet promotes the intake of whole grains, fruits, vegetables, legumes, and non-hydrogenated vegetable oils, such as EVOO. Thus, plant-based diets have low-energy density and high fiber content, which may contribute to CVD prevention, weight loss and long-term body weight maintenance [41,44,88]. Moreover, the profile of fat is mainly MUFA and polyunsaturated fatty acids (PUFA), while SFA intake is lower compared to other dietary patterns. Replacing SFA by MUFA and PUFA has been linked with anti-inflammatory effects and improvements in insulin sensitivity [88]. Among plant-derived foods, the antioxidant effect exerted by several nutrients and bioactive compounds such as vitamin C and E, β-carotenes and polyphenols has been linked to the prevention of CVD and MetS [41,88,95]. Finally, the replacement of some animal-derived foods implies intake restriction of the harmful components mainly present in red and processed meat, such as excessive sodium, heme iron, nitrates and nitrites, which have been linked to CVD outcomes [41,88,96].

In conclusion, recent evidence has demonstrated the protective effect of plant-based diets against MetS, CVD and their individual risk factors. However, healthy plant-derived food choices are crucial to ensure these beneficial effects. Thus, dietary guidelines should consider healthy plant-based dietary patterns as a potential dietary strategy for the prevention and treatment of MetS.

5. Low-Carbohydrate Diet

Low-CH dietary patterns are characterized by a reduction of total CH intake (<50% of daily calorie intake from CH). This type of diet implies a restriction in the intake of several ultra-processed foods, refined grains, starches and foods rich in simple or added sugars [1]. The association between CH intake and the prevalence and management of the MetS is discrepant [97]. In a meta-analysis of 18 studies with 69,554 MetS patients, Lui et al. concluded that the risk of developing MetS was increased in individuals with higher CH intake (2.5% increase in the risk of MetS per 5% energy from CH intake (95% CI 0.4 to 4.8)) [97]. Moreover, some effects on lipid profile were observed in individuals with high CH intake, such as elevated BP, triglycerides and LDL-c and reduced HDL-c levels [98,99]. The mechanisms underlying the health benefits observed in low-CH diets are the avoidance of the rapid absorption associated with some types of CH, such as glucose and refined grains, which leads to an increase in insulin resistance and insulin demand [53,54]. Therefore, in the case of T2DM, recent clinical guidelines do not recommend a specific CH distribution or restriction, and dietary individualization must be prioritized in the treatment and management of this condition [49]. Bazzano et al. conducted a RCT to analyze the effect of a low-CH diet (<40% of total energy intake from CH) compared with a low-fat diet (<30% of total energy from fat, <7% SFA) without energy restriction or physical activity advice in obese adults (BMI 30 to 45 kg/m^2) [50]. After 1 year of intervention, subjects on the low-CH diet without energy restriction showed greater weight loss (−3.5 kg (95% CI −5.6 to −1.4 kg)), specifically in fat mass (−1.5% (CI −2.6% to −0.4%)). Moreover, some cardiovascular risk factors were improved in the low-CH group, such as triglycerides, HDL-c and total cholesterol to HDL-c ratio [50]. Regarding the management of T2DM, low-CH compared to low-fat dietary interventions (<30% of total energy from fat) showed higher reductions of body weight, glycosylated hemoglobin (HbA1c), triglycerides and BP levels and increased HDL-c concentrations and, consequently, a modification in glucose-lowering medications was observed [49,51].

Recent evidence has shown an association between dietary CH intake and the risk of mortality. The Prospective Urban Rural Epidemiology (PURE) study is a cohort study of 135,335 individuals aged 35–70 years from 18 countries from five continents [98]. The aim of this study was to assess the association of dietary fat and CH intake and total mortality and CVD, differentiating this intake according to the profile of FA and CH. The findings of this study suggest the need for an update in dietary guidelines, with emphasis on fat restriction to promote low-fat and CH dietary patterns (around 50–55% of daily energy intake from CH) rich in PUFA and whole-grain CH. Other studies have also observed an association between refined CH intake and a higher risk of cardiovascular events, such as stroke or myocardial infarction [100,101]. However, there is insufficient scientific evidence on low-CH diets (<50–55% of total energy intake) and metabolic improvements have not been demonstrated in order to support or recommend very-low CH diets [98]. Seidelmann et al. observed that with high (>70% of total energy intake) and low (<40%) CH diets the total mortality increased, with 50–55% showing the lowest risk of mortality, representing a U-shaped association [102]. The replacement of CH with other nutrients has shown different effects on total mortality, which was increased in low-CH diets rich in animal-derived fat and/or protein. By definition, low-CH diets promote the restriction of foods rich in CH, such as vegetables, fruits, whole-grain cereals, legumes, etc. Consequently, low-CH diets, compared to diets with 50–55% of energy from CH, showed lower amounts of bioactive compounds such as fiber, PUFAs, polyphenols, vitamins and minerals [103]. Therefore, the use of very low-CH diets as a dietary approach for MetS should promote plant-based fat and/or protein food sources [102].

Among low-CH diets, it has been postulated that very low CH ketogenic diets have a therapeutic role in several NCDs, including overweight and obesity, CVD and MetS [104]. Although there is no standardized definition of the ketogenic diet, it is characterized by a reduction in CH to less than 10% of daily energy intake, which means around 30 to 50 g of CH per day, and a relative increase of fat intake (fat to CH and protein intake ratio of 3:1 to 4:1) [105]. This restrictive dietary pattern has shown protective effects for obesity and CVD by reducing body weight and improving the lipid profile [104,106–108]. The meta-analysis of Bueno et al. observed greater weight loss (weighted mean

difference −0.91 kg (95% CI −1.65 to −0.17 kg)), and reduced triglyceride (−0.18 mmol/L (95% CI −0.27 to −0.08)) and diastolic BP levels (−1.43 mmHg (CI −2.49 to −0.37)), while HDL-c levels increased (0.09 mmol/L (95% CI 0.06 to 0.12)) after following a ketogenic diet compared to a low-fat diet [52]. The mechanisms of action underlying these protective effects are as follows: the absence of dietary CH intake leads to a decrease in insulin secretion, which is translated into an inhibition of lipogenesis and fat accumulation and an increase in lipolysis; a satiety effect of protein intake and its effect on appetite control hormones, such as leptin and ghrelin; and the modulation of insulin secretion and ketone body production which might lead to metabolic improvements, especially in insulin signaling [104,109]. Moreover, CH restriction and the glycogen depletion characteristic of this type of diet lead to the use of ketone bodies as the main source of energy. Nevertheless, energy restriction is necessary to maintain ketone body production. Thus, recent evidence suggests that body weight and CVD benefits observed with ketogenic dietary interventions are due to energy restriction, in spite of the macronutrient distribution of the diet [104,110]. However, health care professionals should consider the difficulties in following a ketogenic diet and the absence of healthy foods such as vegetables, fruits and whole-grain cereals, the intake of which is associated with a lower risk of developing chronic diseases such as CVD, T2DM and some types of cancer.

6. Low-Fat Diet

By definition, the fat content of the low-fat diet comprises less than 30% of total energy, of which <10% are SFA, with a moderate PUFA content and limited *trans* FA [111,112]. In proportion, CH intake is higher and protein intake is moderate (around 15–17% of total energy intake). Low-fat diets usually include foods and products with reduced total fat content, such as low-fat dairy products instead of whole-fat products and derivatives. Low fat diets in weight-loss oriented dietary interventions showed a reduction in the risk of premature mortality in obese adults [56]. In this sense, a meta-analysis of 34 RCTs observed an 18% lower risk of all-cause mortality in weight-loss oriented dietary interventions in obese adults (95% CI 0.71 to 0.95), while no significant effects were observed in CVD mortality or incidence [56]. Recently, a network meta-analysis described the effectiveness of the low-fat diet for body weight reduction compared to the usual dietary advice and dietary patterns after short-term intervention (6 months), with this effect being attenuated after one year [55].

Clinical trials evaluating the effect of a low-fat diet on the prevalence of MetS have shown conflicting results [113–116]. Dietary interventions based on low-fat intake (around 20% of total energy intake from fat) slightly reduced MetS components, but no significant effects were observed for CVD or the incidence of coronary heart disease in postmenopausal women compared to the usual diet [113,114]. Nevertheless, following a low-fat diet was not associated with a lower prevalence of MetS in older subjects at high CVD risk [115]. In this sense, Veum et al. compared the effect of a low-fat, high-CH diet (around 30% of total energy intake from fat) vs. a very high-fat, low-CH diet (around 73% total energy intake from fat) on MetS components [117]. No significant differences were observed in MetS components, body weight and body composition in the medium-term [117]. Similar findings were described by Gardner et al. in the DIETFITS trial, in which both low-fat and low-CH dietary approaches showed significant weight loss with no differences between the two interventions [118].

Regarding BP and blood lipoproteins, a low-fat diet showed beneficial effects on systolic and diastolic BP management, and improved HDL-c and LDL-c levels in the short term compared to usual diet, but these effects were reduced in long-term interventions [33,55]. However, in a meta-analysis of RCTs including 17,230 hypertensive and pre-hypertensive participants, Schwingshackl et al. suggested that the MedDiet and the DASH diet are more effective in long-term BP management compared to low-fat diets [33]. Likewise, low-CH diets showed greater effects on the control of glycated hemoglobin and blood lipid levels than low-fat diets in a short to medium term intervention [52,112,119,120]. In the case of glucose metabolism and insulin control, some RCTs have not identified significant effects of low-fat dietary interventions versus other dietary approaches, while higher triglyceride levels were observed, mostly when simple CH proportion is increased [121–125]. In the case of T2DM management,

Basterra-Gortari et al. found that a low-fat diet did not have an effect on glucose-lowering medication management while the MedDiet supplemented with EVOO could delay its requirement in older people at high CVD risk [126].

MetS is associated with a pro-inflammatory state, and it has been proposed that a low-fat dietary intervention inducing weight loss slightly reduces inflammatory biomarkers such as high-sensitive c-reactive protein (CRP), interleukin−6 (IL) and tumor necrosis factor alpha (TNF-α) levels [16,127–129]. These results are inconclusive, and the effects observed depend on weight loss and diet composition, particularly dietary fiber, fruits and vegetables [16,128]. Additionally, some studies observed that low-fat dietary interventions could improve the microbiome dysbiosis linked to MetS by increasing α-diversity [130,131]. However, limited results are available and more evidence regarding long-term response is needed [132]. Furthermore, nutrigenetic interactions have been described between dietary fat content and metabolic response [133].

Based on the evidence available, current dietary guidelines, such as the 2015–2020 American and European Dietary Guidelines, should avoid stating upper limits of total fat intake, mainly from healthy unsaturated FA. Moreover, this recommendation should include not exceeding 10% of total energy intake from SFA and the replacement of SFA by MUFA and PUFA [22,134].

7. High-Protein Diet

Recent evidence suggests that a high-protein dietary pattern leads to greater weight-loss and CVD improvements than standard protein diets (0.8 g protein/kg body weight). High-protein diets are characterized by a 20–30% of daily energy intake from protein, which means around 1.34 to 1.5 g protein/kg body weight [57]. Currently, the use of high-protein dietary interventions has been postulated for the treatment of obesity, MetS and glycemic control [58,93]. The effect of high-protein dietary strategies for weight management is controversial. A meta-analysis of 18 studies on the effect of a high-protein diet in T2DM patients showed that a high-protein diet did not significantly decrease body weight compared to a regular protein diet [135]. Moreover, no significant effects where observed for glycemic control parameters, such as fasting glucose, insulin and HbA1c, blood lipid profile or BP levels. Nevertheless, a significant reduction of triglyceride levels was observed in participants who followed a high-protein diet. In the case of MetS, high-protein diets with CH restriction have shown effective weight-loss in obese adults with MetS [57,58]. Campos-Nonato et al. performed a RCT in 118 adults with MetS to evaluate the effect of a hypocaloric high-protein diet compared to a hypocaloric standard protein diet (500 kcal/day less than the metabolic rate and 1.34 g protein/kg body weight or 0.8 g protein/kg body weight, respectively) [57]. Weight-loss after 6 months of the dietary interventions was significantly higher in participants who followed a high-protein diet (−7.0 kg ± 3.7; p-value = 0.046) compared to the standard protein diet (−5.1 kg ± 3.6; p-value = 0.157) [57]. MetS criteria, including fasting blood glucose, insulin, homeostatic model assessment for insulin resistance (HOMA-IR) index, and triglyceride, and cholesterol levels, improved in both intervention arms, but non-significant differences were observed in the comparison between groups. The Optimal Macronutrient Intake Trial to Prevent Heart Disease (OmniHeart) study was a randomized, controlled, three-period, crossover nutritional study with 164 participants with overweight or obesity and prehypertension or stage 1 hypertension free of T2DM [136]. This study aimed to evaluate insulin sensitivity with the quantitative insulin sensitivity check index among three dietary interventions: a high-CH diet (58% of daily kcal from CH; 15% from protein and 27% from fat); a protein diet (replacement of 10% of total CH to protein, 25% of daily kcal intake from protein, mainly from plant-based protein sources); and an unsaturated diet (replacement of 10% of total CH to unsaturated fat, 37% of daily kcal intake from fat, mainly from seeds and oils such as olive, canola and safflower oils and nuts). The protein and high-CH dietary patterns did not affect insulin sensitivity, while the unsaturated diet showed improvements in insulin sensitivity, suggesting that the replacement of CH by unsaturated fat, such as in the MedDiet patterns, are alternative dietary approaches to improve insulin sensitivity. The mechanism underlying the potential health benefits of a high-protein diet is that protein induces satiety, which is translated into

reduced energy intake in the next meals [137,138]. Furthermore, high protein intake avoids muscle mass loss during energy-restrictive dietary interventions for weight loss [139].

Among protein food sources, meat and meat derived products have been associated with a higher risk of developing T2DM, CVD and MetS [12,13,140]. Dietary guidelines recommend prioritizing plant-based protein food sources such as soy, legumes, beans, nuts and seeds instead of meat and processed meat [21]. Plant-based protein food sources are rich in fibre, phenolic compounds and PUFA, while cholesterol, trans or SFA are in lower proportions [141]. In a recent meta-analysis of 36 RCTs, red meat consumption had no effect on the blood lipid profile or BP, while after analyses stratified by the type of comparison diet, the substitution of red meat with plant-based protein foods showed a reduction in total cholesterol and LDL-c levels [93]. Thus, strong evidence promotes the intake of plant-based protein food sources, and this should also be recommended to promote environmental sustainability.

8. Other Dietary Patterns and Strategies

Other dietary alterations have been shown to improve the MetS condition, such as the Nordic Diet, which is characterized by a high content of whole-grain high-fiber products (such as rye, barley, oat, rice, vegetables, fruits and nuts), with rapeseed oil as the main source of dietary fat and a high intake of fish and shellfish [142,143]. Similar to the DASH and the MedDiet, the Nordic diet is considered to be a healthy dietary pattern in that it promotes the intake of vegetables, fruits, fish, poultry, nuts, and is low in sodium, red meat and processed foods. A recent meta-analysis of 5 RCTs including 513 participants demonstrated the effectiveness of the Nordic diet in improving some MetS criteria, mainly systolic and diastolic BP (weighted mean differences −3.97 mmHg (95% CI −6.40 to −1.54; $p < 0.001$); −2.08 mmHg (95% CI −3.43 to −0.72; $p = 0.003$), respectively) [59]. Moreover, improvements in LDL-c (0.30 mmol/l (95% CI −0.54 to −0.06; $p = 0.013$)), but not in HDL-c and TG levels, were observed compared to control diets [59]. Further studies are needed to evaluate the beneficial effects of the Nordic diet on MetS management and prevention.

Among dietary strategies, intermittent fasting has shown benefits for CVD, T2DM, metabolic disturbances and cancer, mainly because of the daily caloric restriction involved [63]. The main cardiometabolic effects observed after an intermittent-fasting intervention are weight loss and improvements in insulin resistance, dyslipidemia, BP levels and inflammation [60–62]. Despite the evidence and potential health benefits of intermittent fasting, the applicability of this dietary strategy is complex and trained health care providers are needed to avoid side effects. Furthermore, De la Iglesia et al. postulated other potential dietary approaches for the prevention and treatment of MetS, such as diets rich in omega−3 FA, low glycemic index, high antioxidant capacity or high meal frequency dietary interventions [144].

Thus, dietary intervention based on energy restriction, independently of the distribution of macronutrients, might influence BP and CVD. Accordingly, most scientific evidence highlights the relevance of dietary quality rather than quantity, especially in the management and prevention of MetS [8,145–147]. Moreover, the effectiveness of every dietary intervention is associated with the previous metabolic state (e.g., presence of insulin resistance, T2DM, altered fasting glucose levels, etc.) [148,149]. While multifaceted lifestyle interventions focus on weight-loss and the promotion of physical activity, adherence is the key factor in achieving the beneficial effects observed in each dietary pattern, with intervention adherence being decisive in the results observed independently of the type of diet [150–152].

9. Conclusions

The protective effects of healthy dietary patterns on MetS seem to be due to the sum of small dietary changes rather than the restriction of any single nutrient. On comparing low-fat diets and very-restricted diets, the scientific evidence supports the use of the MedDiet intervention as the new paradigm for MetS prevention and treatment. The nutritional distribution and quality of the MedDiet allows health professionals to provide easy-to-follow dietary advice without the need for a restricted

diet. Nonetheless, RCTs on the effects of a low-CH MedDiet style diet, promoting the intake of whole grain and plant-based protein food sources in patients with MetS, are needed to demonstrate the efficacy of this dietary pattern.

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Review

The Legacy Effect in the Prevention of Cardiovascular Disease

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Abstract: The "legacy effect" describes the long-term benefits that may persist for many years after the end of an intervention period, involving different biological processes. The legacy effect in cardiovascular disease (CVD) prevention has been evaluated by a limited number of studies, mostly based on pharmacological interventions, while few manuscripts on dietary interventions have been published. Most of these studies are focused on intensive treatment regimens, whose main goal is to achieve tight control of one or more cardiovascular risk factors. This review aims to summarise the legacy effect-related results obtained in those studies and to determine the existence of this effect in CVD prevention. There is sufficient data to suggest the existence of a legacy effect after intensive intervention on cardiovascular risk factors; however, this effect is not equivalent for all risk factors and could be influenced by patient characteristics, disease duration, and the type of intervention performed. Currently, available evidence suggests that the legacy effect is greater in subjects with moderately-high cardiovascular risk but without CVD, especially in those patients with recent-onset diabetes. However, preventive treatment for CVD should not be discontinued in high-risk subjects, as the level of existing evidence on the legacy effect is low to moderate.

Keywords: legacy effect; metabolic memory; cardiovascular disease; diet; diabetes; hypertension; dyslipidaemia

1. Introduction

Cardiovascular disease (CVD) has emerged as a major cause of morbidity and mortality, accounting for 30% of all deaths worldwide. The intensive management of cardiovascular risk factors is required to reduce its incidence, and some authors suggest that achieving tight control at early stages of the disease, before vascular damage has developed, is a determinant of outcomes [1].

The legacy effect concept refers to long-term sustained benefits after a period of intensive treatment intervention, even after cessation of the intervention [2]. Initially described in diabetic patients, it has also been observed in patients with hypertension or hypercholesterolemia [3]. Moreover, the concept of metabolic memory, mostly described in the study of diabetic models, refers to DNA's ability to store information related to prior poor metabolic control; for example, persistent adverse effects of hyperglycaemia may reduce the potential benefit of subsequent improvements in glucose control, and induce the development of vascular complications in target organs [4,5]. Therefore, achieving good glycaemic control in the early stages of diabetes could be critical in preventing late-stage complications [6,7].

Metabolic memory was first described in 1987 when Engerman et al. observed a higher incidence of diabetic retinopathy among dogs in which good glycaemic control was preceded by a longer period

of abnormal glucose levels [8]. Likewise, long-term overproduction of fibronectin, both by endothelial cells cultured in a high-glucose medium and by the kidneys of streptozocin-induced diabetic rats after the restoration of near-normoglycemia, was also detected [9]. Further investigation in 1993 showed that transplanting the islets of Langerhans shortly after diabetes mellitus onset in rats could reverse diabetic retinopathy [10].

However, most studies examining the legacy effect on CVD and its related risk factors for vascular complications are based on pharmacological interventions, with low emphasis on dietary interventions; thus, the long-term effects of strict dietary regimens remains unknown. In this review, we focus on the recent evidence of the legacy effect and metabolic memory in CVD development after intensive pharmacological and non-pharmacological interventions addressing cardiovascular risk factors.

2. Methods

The aim of this narrative review was to assess those scientific studies that evaluated the existence of the legacy effect in the prevention of CVD after a nutritional or pharmacologic intervention. We searched for scientific studies published in the last ten years and written in English in PubMed Medline Database by using specific search terms ("legacy effect", "metabolic memory", "cardiovascular disease", "diet", "diabetes", "hypertension" and "dyslipidemia"). In addition, recent reviews and meta-analysis about the legacy effect, metabolic memory and its pathophysiological mechanisms were also included. To sensitise the search and select the best articles, we used the aforementioned keywords and applied different Boolean operators. Finally, a total of 64 articles were selected for this review. Most of them were clinical trials in humans, and there was also a small proportion performed in animal models. The differences observed among the outcomes of the trials and their post-trial follow-up studies have been described by using the difference in means, risk ratio (RR), odds ratio (OR) and hazard ratio (HR) measures. The publication, attrition and co-intervention bias, and the baseline characteristics of the patients included in the trials should be taken into consideration for the interpretation of the results.

3. Pathophysiological Mechanisms Involved in Metabolic Memory

Multiple mechanisms have been described as relevant to the development of the legacy effect. Diabetic models have been used to thoroughly study the concept of metabolic memory. Furthermore, studies performed on hypertension models have shown that similar mechanisms are involved in the maintenance of beneficial post-intervention effects.

3.1. Oxidative Stress

Chronic hyperglycaemia induces uncoupling of the mitochondrial electron transfer chain in endothelial cells, causing excessive production of superoxide anion and other reactive oxygen species (ROS), thereby increasing cellular oxidative stress and inducing endothelial dysfunction. Moreover, ROS can cross membranes and damage macromolecules (nucleic acids, mitochondrial DNA and proteins), many of which may have longer half-lives, and hence may exert metabolic effects. These oxidative stress markers can persist in endothelial cells despite glucose normalisation after prolonged hyperglycaemia, suggesting a metabolic memory phenomenon (Figure 1) [4,5]. Furthermore, postprandial glucose oscillations in diabetic patients have been associated with increased ROS marker levels and vascular stress; the cells' inability to adapt to this dynamic environment leads to cell damage and elevated collagen and fibronectin levels that remain abnormal for several days after glucose levels have normalised [11–13]. In hypertension models, the overproduction of ROS by NADPH oxidase upregulation due to renin-angiotensin system activation appeared to persist after cessation of the hypertensive period and was related to deleterious effects. Otherwise, the intensive blocking of the renin-angiotensin system showed a long-term reduction in the overproduction of ROS [14].

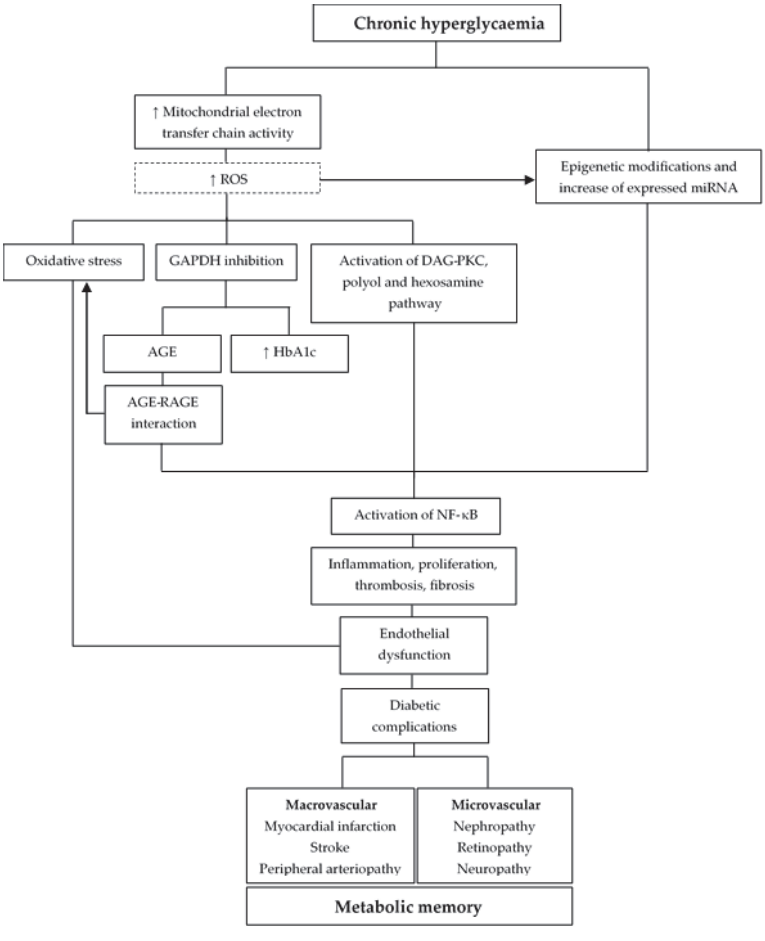

Figure 1. Pathophysiological mechanisms of metabolic memory in chronic hyperglycaemic conditions. Abbreviations: AGE: advanced glycation end-products; DAG-PKC: diacylglycerol-protein kinase C; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HbA1c: glycated haemoglobin; miRNA: micro RNA; NF-κB: nuclear factor κB; RAGE: AGE receptor; ROS: reactive oxygen species.

3.2. Non-Enzymatic Glycosylation of Proteins and Chronic Inflammation

Superoxide anion inhibits glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity, leading to the accumulation of all glycolytic intermediates, which can react non-enzymatically with proteins, lipids, and nucleic acids to form advanced glycation end-products (AGEs). AGEs may accelerate ageing processes, and they cannot be degraded easily by the enzymes involved in normal metabolism. In addition, the most exposed proteins, such as haemoglobin, are also highly glycated [4]. AGEs also promote oxidative stress, vascular hyperpermeability, pathological angiogenesis, and thrombogenic reactions via interaction with their receptors (RAGEs), which promote endothelial dysfunction and atherosclerosis [5,15,16]. Hyperglycaemia also causes the activation of the diacylglycerol (DAG)-protein kinase C (PKC) pathway, fructose production, and increased flux through the hexosamine pathway, which intensifies the expression of various adhesive molecules, proinflammatory cytokines, and growth factors. These mechanisms activate nuclear factor κB (NF-κB), a rapid-response transcription factor involved in inflammatory reactions and proapoptotic programs in diabetes.

Even after glycaemic control has been achieved, AGEs may be used as markers to measure cumulative diabetic exposure, especially in those with vascular complications, whereas intensive treatment is associated with significantly lower AGE levels. These pathways are responsible for metabolic memory and contribute to the development and progression of CVD and diabetic complications. Glycaemic memory can be measured by glycated haemoglobin (HbA1c), although AGEs have also been correlated with retinopathy progression, independent of HbA1c level [16]. Some medications could alter these pathways and, consequently, prevent the development of CVD. Metformin, pioglitazone, glucagon-like peptide-1 (GLP-1) receptor agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors have been shown to prevent AGE formation or decrease inflammation by blocking AGE and NF-κB pathways, reversing the metabolic memory phenomenon [12,16–18]. Also, angiotensin-converting-enzyme inhibitors (ACEI) and angiotensin II receptor type 1 (AT-1) blockers can reduce AGE formation and, subsequently, the inhibition of superoxide generation [17]. It is widely recognised that benefits of renin-angiotensin-aldosterone system (RAAS) inhibition extend beyond blood pressure (BP) reduction and may last even after switching from an intensive BP-lowering strategy to a conventional one; its effectiveness in preventing diabetic complications indirectly suggests that RAAS dysregulation is involved in triggering organ damage in diabetic patients [19].

3.3. Epigenetic Modifications

Chronic hyperglycaemia can induce epigenetic modifications, which could underlie endothelial dysfunction and the development of metabolic memory in diabetic complications. Recent research suggests that DNA methylation and post-translational histone modifications in a hyperglycaemic environment could lead to enhanced expression of proinflammatory genes, such as the increased expression of the key p65 subunit of NF-κB in various target tissues, which persists long after restoration of normoglycemia. Moreover, in cell and animal models exposed to transient-high and subsequent normal glucose levels, an increase in differentially expressed miRNAs has been observed; these may play a role as regulators of endothelial dysfunction in metabolic memory by increasing the constitutive activation of NF-κB pathway. However, the potential reversal of glucotoxicity and lipotoxicity depends on how long the patient has been exposed to this poor metabolic environment [20,21].

4. Legacy Effect after Dietary Intervention

Few studies have assessed the legacy effect after dietary intervention. Most of these have been done in animals. Below, we describe the most relevant findings.

One study showed that mice switched from dietary restriction (DR) to ad libitum (AL) intake had significantly better glucose tolerance at 6–10 months compared to the AL group, suggesting that there was positive glycaemic memory in the DR group [22–24]. Moreover, it was also observed that short-term reversion to a normal diet in rats after initial exposure to a high-fat diet (HFD) could not restore the insulin resistance-induced complications. However, administering metformin to these animals induced remarkable amelioration of anomalies associated with insulin resistance and endothelial dysfunction via lipotoxicity reduction [25,26]. In another study, mice fed a HFD (60% kcal from fat) for 21 weeks were compared to those fed a low-fat diet (LFD, 10% kcal from fat); the HFD animals developed type 2 diabetes mellitus (T2DM) and gained much more weight compared to mice fed an LFD. During the 4-week intervention period following diabetes onset, insulin-treated mice maintained on an HFD exhibited significantly improved glucose tolerance test results compared to sham-treated animals. However, mice remaining on a HFD following cessation of the insulin treatment exhibited no benefit from the early insulin therapy and continued to gain weight, had worse glucose tolerance test results, and displayed significantly higher fasting insulin, C-peptide, and leptin levels compared to sham-untreated controls, and early insulin therapy in mice maintained on the LFD. In fact, early insulin therapy only conferred a beneficial effect in animals switched to an LFD after the insulin treatment. These findings indicated that the legacy effect of early insulin-treatment was only observed

in diabetic mice switched to an LFD after the insulin treatment and emphasised the vital role of diet adherence in diabetes control at any stage of disease progression [27].

The legacy effect was also confirmed in rats subjected to an HFD, when time-restricted feeding was alternated with AL feeding, with no significant differences in body weight observed between the alternated feeding and the control group (AL feeding only) in a short term-study (12 weeks). However, in the long-term study (25 weeks), when mice were maintained on time-restricted feeding for 13 weeks and then switched to AL feeding for 12 weeks, they showed significantly less body weight gain after returning to an HFD compared to the control group, which were maintained on time-restricted feeding throughout the 25 weeks (112% versus 51% body weight increase, respectively) [28]. Further research performed on male rats showed that maintaining an HFD induced persistent changes in sperm cells that could be transmitted to the female descendants, impairing their glucose tolerance and insulin secretion [29].

To our knowledge, there is limited evidence assessing the existence of a legacy effect in humans after dietary intervention. The effects of caloric restriction (approximately 25% of daily energy intake) or intermittent fasting (up to 18 h/day) in humans have been demonstrated mainly in observational studies, and only one clinical trial, the Comprehensive Assessment of the Long-term Effects of Reducing Energy Intake (CALERIE) study, was performed. Both strategies improve general health indicators and slow or reverse ageing and disease processes, such as obesity, insulin resistance, dyslipidaemia, hypertension and inflammation, although intermittent fasting seems to exert a greater effect. Improvements in health indicators typically begin within the first month, after the start of intermittent fasting, and then dissipate over a period of several weeks after resumption of a normal diet [30]. This effect was also evaluated in a small clinical trial of 24 obese older patients (mean age 65–79 years) after caloric restriction (CR) intervention plus resistance training (RT) compared to RT intervention for five months. Despite clinically meaningful weight loss and concurrent favourable shifts in total body and thigh composition in the CR+RT group compared to RT group during the intervention period, these improvements were generally not sustained over the long-term (18 months). At the end of the study, only weight was significantly lower compared to baseline in the CR+RT group. Therefore, this trial showed a temporary legacy effect which lost intensity during the follow-up [31].

The Oslo cardiovascular study was a five year randomised intervention conducted in healthy middle-aged men at high risk of coronary heart disease (CHD) assigned to a dietary advice group (main objectives: to reduce daily intake of saturated fats, sugar and alcohol; to increase daily intake of fish, vegetables and fruit; to reduce weight; to quit smoking) compared to a control group. At 40 years of follow-up, a significant reduction (19%) in the risk of death at first myocardial infarction (MI) was detected in the intervention group (HR 0.71, 95% confidence interval (CI), 0.51–1.00), with no significant difference in total mortality. The legacy effect was achieved through the dietary intervention (as evidenced by lower cholesterol and serum triglyceride levels and weight reduction) because few men quit smoking; therefore, the effect of this measure was small [32].

In the Look Action for Health in Diabetes (Look-AHEAD) trial, overweight/obese patients with T2DM were randomly assigned to an intensive lifestyle intervention to achieve and maintain a weight loss ≥7%, which included: hypocaloric diet (1200–1800 kcal/day; less than 30% of daily energy intake from fat and less than 10% from saturated fat; at least 15% of daily energy intake from protein), physical activity and diabetes education, compared to standard care for one year. Patients included in the intensive lifestyle intervention had greater weight reduction with better control of cardiovascular risk factors (glycaemic control, systolic BP and lipid profile), and more improvement in fitness levels throughout the four year follow up period compared to the control group [33].

5. Legacy Effect in Diabetic Patients after Intensive Glycaemic Control

Several clinical trials have assessed legacy effects in different populations of patients with diabetes, the main results of which are listed below. The specific data from each article are shown in Table 1.

Table 1. Studies assessing the legacy effect after intensive glycaemic control.

	Glycaemic Control				
	DCCT/EDIC (*n* = 1.441/1.394) *	**UKPDS (*n* = 4.209/3.277) ***	**ACCORD (*n* = 10.251/8.601) ***	**VADT (*n* = 1.791/1.655) ***	**ADDITION (*n* = 153,107 Screened/125,083 Unscreened) ***
In-trial follow-up duration/total follow-up duration	6.5 years/17 years	10 years/20 years	3.7 years/8.8 years	5.6 years/15 years	5.3 years/10 years
Patient characteristics	Mean age 27 years; T1DM	Mean age 56.4 years; newly diagnosed T2DM	Mean age 62.2 years; long-standing duration T2DM (10 years)	Military veterans; mean age 60.4 years; poorly controlled and long duration T2DM (11.5 years)	Mean age 59.9 years; newly diagnosed T2DM after regular screening
Type of intervention	Intensive vs. standard therapy (glycaemic target 70–120 mg/dL vs. no goals)	Intensive vs. standard therapy (sulfonylurea/insulin/metformin vs. DR)	Intensive vs. standard therapy (HbA1c target <6% vs. <7.9%)	Intensive vs. standard glucose control (HbA1c targets <6% vs. <9%)	Intensive vs. routine diabetes care (HbA1c target <7%)
Dietary intervention	Standard care	3-month diet in both groups. DR in control group	Standard care	Standard care	Standard care
In-trial outcomes	Reduction in retinopathy, neuropathy and development of microalbuminuria in the intensive treatment group	Reduction in microvascular complications, MI and total mortality in the intensive-therapy group	Reduction in nonfatal MI and increased cardiovascular and total mortality in the intensive-therapy group	Significant reduction in progression to microalbuminuria in the intensive-therapy group	Reduction in fatal and nonfatal CVD
Legacy effect	Yes	Yes	No	No	Yes
Effects in the post-trial follow-up	Reduction in the risk of nephropathy, nonfatal MI, stroke and cardiovascular death with intensive treatment	Reduction in microvascular disease (maintained in the sulfonylurea-insulin group), MI and death from any cause in the intensive-therapy group	A trend to reduction in nonfatal MI, nonfatal stroke and death from any cause, and persisted increase in death from cardiovascular causes in the intensive-therapy group	Reduction in cardiovascular events and a trend to reduction in cardiovascular and total mortality in the intensive-therapy group after 10 years of follow-up	Significantly lower risk in all-cause mortality and cardiovascular events

Abbreviations: CVD: cardiovascular disease; DR: dietary restriction; HbA1c: glycated haemoglobin; MI: myocardial infarction; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; DCCT: Diabetes Control and Complications Trial; EDIC: Epidemiology of Diabetes Interventions and Complications; UKPDS: United Kingdom Prospective Diabetes Study; ACCORD: Action to Control Cardiovascular Risk in Diabetes; VADT: Veterans Affairs Diabetes Trial; ADDITION: Anglo-Danish-Dutch study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care. * The number of randomised patients enrolled in the interventional phase and the cohort of post-trial follow-up, respectively.

The Diabetes Control and Complications Trial (DCCT) was performed in patients with type 1 diabetes mellitus (T1DM), who were randomly assigned to either intensive or standard glycaemic control regimens. After 6.5 years of intervention, the development of severe proliferative or non-proliferative retinopathy (HR 0.47, 95% CI, 0.14–0.67), clinical neuropathy (HR 0.60, 95% CI, 0.38–0.74), and microalbuminuria (HR 0.39; 95% CI, 21–52) was significantly lower in the intensive treatment group [34]. After the DCCT trial, patients were followed up for 17 years in the Epidemiology of Diabetes Interventions and Complications (EDIC) study. A legacy effect was observed in the former intensive-therapy group during this period, as evidenced by a significant reduction in the prevalence of cardiovascular events (nonfatal MI, stroke, or death from CVD; HR 0.43; 95% CI, 0.12–0.79), and nephropathy (HR 0.54, 95% CI, 0.34–0.84) (Table 1) [35].

The United Kingdom Prospective Diabetes Study (UKPDS) recruited patients with newly diagnosed T2DM (six months from diagnosis); after a three-month diet (main features: low and moderately high daily intake of saturated fat and fibre, respectively; about 50% of daily caloric intake from carbohydrates; hypocaloric diet in overweight patients), they were randomised to intensive glucose control with medication (sulfonylurea, insulin, metformin) or to conventional dietary treatment, and they were followed-up for ten years. Intensive glucose management was associated with a significant reduction for any diabetes-related endpoint (12%) and for any diabetes-related death (10%) compared to the control group. Most of the risk reduction in the "any diabetes-related aggregate" endpoint was due to a 25% risk reduction in microvascular endpoints. However, there was no significant reduction in macrovascular endpoints between both arms of the study. Moreover, patients in the intensive group had more hypoglycaemic episodes than those in the conventional one [36]. Although differences between both groups in HbA1c levels were lost after the first year, a clearly different incidence in clinical endpoints in the post-trial follow-up of the UKPDS was observed. Thus, relative reductions in risk persisted at ten years for any diabetes-related endpoint (RR 0.91, CI 95%, 0.83–0.99), for microvascular disease (RR 0.76, CI 95%, 0.64–0.89), MI (RR 0.85, CI 95%, 0.74–0.97), and for death from any cause (RR 0.87, CI 95%, 0.79–0.96), and emerged over time in the intensive therapy group, as more events occurred. In the metformin group (overweight patients), significant risk reductions persisted for any diabetes-related endpoint (RR 0.79, CI 95%, 0.66–0.95), MI (RR 0.67, CI 95%, 0.51–0.89), and death from any cause (RR 0.73, CI 95%, 0.59–0.89) (Table 1) [37,38].

The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial included patients with T2DM with a mean duration of ten years and previous cardiovascular events or multiple cardiovascular risk factors. The patients were assigned to receive intensive or standard therapy (targeting HbA1c <6% versus vs. <7.9%, respectively) for 3.7 years, with a combination of different hypoglycaemic drugs including insulin [39]. It was not observed statistically significant benefit during the intervention period nor after ending the trial in the intensive treatment group, except for the rate of nonfatal MI, which was lower than in the standard therapy group (HR 0.76, 95% CI, 0.62–0.92). In fact, a significant increase in cardiovascular mortality (HR 1.35, 95% CI, 1.04–1.76), and death from any cause (HR 1.22, 95% CI, 1.01–1.46) was detected during and after the ACCORD trial in the intensive treatment group, attributed to a higher incidence of severe hypoglycaemia in those patients (Table 1) [39,40].

The Veterans Affairs Diabetes Trial (VADT) was performed in patients with poorly controlled long-standing T2DM (mean duration of 11.5 years), 40% of whom had already suffered a cardiovascular event. They were randomly assigned to intensive or standard therapy for a median of 5.6 years. Other cardiovascular risk factors were treated uniformly. The intensive glucose-lowering treatment led to a between-group difference of 1.5 percentage points in HbA1c levels (6.9% in the intensive-therapy group vs. 8.4% in the standard-therapy group) during the in-trial period. The only benefit observed was a slower progression of microalbuminuria in the intensive treatment group. However, a significantly lower incidence of CVD (HR 0.83, 95% CI, 0.70–0.99) in patients originally assigned to intensive therapy was observed after ten years of follow-up. Nevertheless, over a 15-year follow-up period, the risks of major cardiovascular events (HR 0.91, 95% CI, 0.78–1.06), or death from any cause (HR 1.02, 95% CI, 0.88–1.18) were not lower in the intensive-therapy group than in the standard therapy group,

suggesting a modest and transient long-term cardiovascular benefit of intensive glucose-lowering therapy in patients with more advanced diabetes (Table 1). In fact, the cardiovascular benefit (legacy effect) of intensive therapy lasted when HbA1c curves of both groups were separated (Table 1) [41–43].

The Anglo-Danish-Dutch study of Intensive Treatment in People with Screen-Detected Diabetes in Primary Care (ADDITION) study recruited middle-aged adults with a moderate to high risk of T2DM for diabetes screening, in order to evaluate the risk of CVD and mortality among incident cases of T2DM in a screened group compared to an unscreened population. Participants diagnosed with T2DM were offered intensive multifactorial treatment and compared to those receiving routine care for five years, with a reduction in fatal and nonfatal CVD of 17% shown. After a 10-year follow-up, patients diagnosed after population-based screening had a significant 21% lower risk in all-cause mortality (HR 0.79, 95% CI, 0.74–0.84) and a significant 16% lower risk in cardiovascular events (HR 0.84; 95% CI, 0.80–0.89), but no significant risk reduction on cardiovascular mortality compared to unscreened patients [44]. Moreover, population-based diabetes screening was associated with less need for insulin therapy after ten years and slightly better long-term glycaemic control compared to patients diagnosed during usual care (Table 1) [45].

The Diabetes and Aging Study was a large observational study of newly diagnosed T2DM patients and followed those with long survival post-diagnosis (≥10 years). The study assessed the impact of HbA1c levels ≥6.5% in the first year after T2DM diagnosis on later CVD risk. The risk of micro- and macrovascular events was higher in those patients with HbA1c levels ≥6.5% compared to those with lower levels, whereas mortality risk was significantly higher in patients with HbA1c levels ≥8%. These results suggest it is necessary to achieve normoglycemia in the first 12 months after T2DM diagnosis to generate a legacy effect (Table 1) [46].

6. Legacy Effect after Blood Pressure Control

Several clinical trials have assessed legacy effects in different populations of hypertensive patients, the main results of which are listed below. The specific data from each article are shown in Table 2. A separate UKPDS post-trial follow-up study assessed whether risk reductions for micro- and macrovascular complications were achieved and maintained in a subgroup of hypertensive subjects with newly diagnosed T2DM with tight versus less-tight BP control with captopril or atenolol. However, differences in BP between groups (tight vs. less tight) disappeared within two years after trial termination, and most cardiovascular benefits were not sustained during the 10-year post-trial follow-up period. Only a reduced risk for peripheral vascular disease associated with tight BP control was significant (RR 0.50, 95% CI, 0.28–0.92). Hence it would be necessary to maintain good BP levels over time to preserve this previous benefit (Table 2) [36,47].

The Systolic Hypertension in the Elderly Program (SHEP) trial was performed in elderly patients with isolated systolic hypertension who were randomised to chlortalidone therapy or placebo (plus atenolol or matching placebo, if BP remained uncontrolled). Over a mean follow-up of 4.5 years therapy, reduction in fatal or nonfatal stroke (RR 0.64, 95% CI, 0.50–0.82), MI (RR 0.67, 95% CI, 0.47–0.96), and heart failure (RR 0.51, 95% CI, 0.37–0.71) in the chlortalidone group compared to placebo was observed. However, there was no significant risk reduction in all-cause and cardiovascular mortality. After a 22-year follow-up, significant, albeit modest life expectancy gains free from CVD-related deaths were observed in the chlortalidone group, corresponding with approximately one day (HR 0.89, 95% CI, 0.80–0.99) gained for each month of treatment. (Table 2) [48].

Table 2. Studies assessing the legacy effect after intensive BP control.

	Blood Pressure Control					
	HDS (UKPDS) (*n* = 1.148/884) *	**SHEP (*n* = 4.736/1.885) ***	**ALLHAT (*n* = 32.804/27.755) ***	**ROADMAP (*n* = 4.447/1.758) ***	**ANBP2 (*n* = 6.083/5.378) ***	**ASCOT (*n* = 8.580/7.302) ***
In-trial follow-up duration/Total follow-up duration	4 years/10 years	4.5 years/22 years	4.9 years/8–13 years	3.2 years/6.5 years	4.1 years/10 years	5.5 years/16 years
Patients characteristics	Mean age 56.4 years; newly diagnosed T2DM and hypertension	Mean age 71.6 years; isolated systolic hypertension (≥160 mm Hg) with no pre-existing CVD	Mean age 66.9 years; ≥55 years, hypertensive and at least one additional risk factor for CHD events	Mean age 61.2 years; T2DM with cardiovascular risk factors and normoalbuminuria	Mean age 71.8 years; average systolic BP of 160 mm Hg or average diastolic BP of 90 mm Hg, without recent cardiovascular events	Mean age 64 years; hypertension with at least three other cardiovascular risk factors without CHD
Type of intervention	Tight vs. less tight BP control with captopril or atenolol (target <150/85 vs. <180/105 mm Hg)	Chlortalidone vs. placebo	Amlodipine, lisinopril or doxazosin vs. chlortalidone	Olmesartan vs. placebo	ACE inhibitor vs. diuretic therapy	Amlodipine-based regimen vs. atenolol-based regimen
Dietary intervention	Standard care	Standard care	Standard care	Standard care	Standard care	Standard care
In-trial outcomes	Reduction in MI, sudden death and microvascular complications	Reduction in fatal or nonfatal strokes, MI and heart failure	No differences in CHD or nonfatal MI	Increased time to the onset of microalbuminuria	Less risk of cardiovascular and all-cause mortality in control group	Reduction in cardiovascular events and mortality in amlodipine-based group
Legacy effect	No	Yes	No	Yes	No	Yes
Effects in the post-trial follow-up	Reduction in peripheral vascular disease in the tight BP control	Approximately one day gained in life expectancy free from cardiovascular death for each month of active therapy in the chlortalidone group	Higher hospitalised and fatal heart failure on amlodipine group and higher stroke mortality on lisinopril group	Significant reduction in CHF, retinopathy and non-significant reduction in the onset of microalbuminuria in the olmesartan group	No differences in cardiovascular outcomes or all-cause mortality	Significant fewer stroke deaths on amlodipine-based group

Abbreviations: ACE: angiotensin-converting enzyme; BP: blood pressure; CHD: coronary heart disease; CHF: congestive heart failure; CVD: cardiovascular disease; MI: myocardial infarction; T2DM: type 2 diabetes mellitus; HDS: Hypertension in Diabetes Study; UKPDS: United Kingdom Prospective Diabetes Study; SHEP: Systolic Hypertension in the Elderly Program; ALLHAT: Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial; ROADMAP: Randomised Olmesartan And Diabetes MicroAlbuminuria Prevention; ANBP2: Second Australian National BP study; ASCOT: Anglo-Scandinavian Cardiovascular Outcomes Trial. * The number of randomised patients enrolled in the interventional phase and the cohort of post-trial follow-up, respectively.

In the Antihypertensive and Lipid Lowering Treatment to Prevent Heart Attack Trial (ALLHAT), 32,804 hypertensive patients with at least another cardiovascular risk factor were randomised to receive chlorthalidone, amlodipine, or lisinopril for 4 to 8 years, and thereafter, passive surveillance continued for a total follow-up of 8 to 13 years. During the in-trial period, no statistically significant differences in CHD or nonfatal MI were observed. In the post-trial follow-up, the only significant differences were observed in secondary outcomes, such as heart failure and stroke mortality, which were higher with amlodipine and lisinopril, respectively, compared to chlorthalidone. However, a significant treatment-by-race interaction was detected and, after accounting for multiple comparisons, none of these results were deemed significant (Table 2) [49].

The Randomised Olmesartan And Diabetes MicroAlbuminuria Prevention (ROADMAP) trial included patients with T2DM with at least one additional cardiovascular risk factor and normoalbuminuria. They were randomly assigned to olmesartan or placebo for 3.2 years; the main outcome was significantly delayed microalbuminuria onset in the olmesartan group. After a 6.5-year observational follow-up period, researchers concluded that congestive heart failure (CHF) requiring hospitalisation (OR 0.23, CI 0.06–0.85) and diabetic retinopathy (OR 0.34, CI 0.15–0.78) were significantly lower in the former olmesartan group, indicating a legacy effect in these outcomes (Table 2) [50,51].

The Second Australian National BP study (ANBP2) observed a lower risk of cardiovascular (HR 0.47, 95% CI, 0.27–0.81) and all-cause mortality (HR 0.63, 95% CI, 0.46–0.86) in the "treatment-naive" group (participants without BP-lowering medication at study registration) compared to BP-lowering medication or "previous treatment" group (BP-lowering treatment at registration; median duration of previous therapy was five years) during the in-trial period in an elderly cohort. No differences were found between groups with respect to randomised treatment allocation to either ACEI or diuretic-based regimens; likewise, there were no differences in cardiovascular outcomes or all-cause mortality when the data from the in-trial and ten-year post-trial follow-up were combined (Table 2) [52].

The Anglo-Scandinavian Cardiovascular Outcomes Trial (ASCOT) enrolled patients with hypertension and at least three other cardiovascular risk factors. In the BP-lowering arm, patients were randomised to either an amlodipine-based regimen (adding perindopril as required) or an atenolol-based regimen (adding bendroflumethiazide and potassium as required). The in-trial results demonstrated a significant reduction in cardiovascular events, mortality and all-cause mortality in subjects assigned to the amlodipine-based group, but no overall difference in cardiovascular mortality (HR 0.90, 95% CI, 0.81–1.01) among treatments. These results could suggest that not all hypotensive drugs are equally effective in preventing CVD, although the resulting BPs are similar. After 16 years of total follow-up (ASCOT Legacy Study), there was no overall difference in all-cause mortality between treatments, although significantly fewer deaths from stroke (HR 0.71, 95% CI, 0.53–0.97) occurred in the amlodipine-based treatment group (Table 2) [53].

Finally, a systematic review and meta-analysis of three clinical trials were performed to analyse whether early versus late initiation of antihypertensive treatment was better at reducing cardiovascular morbidity and mortality. The trials involved 4746 mildly hypertensive (systolic BP 140–159 mmHg) middle-aged patients free of CVD at baseline and with low cardiovascular risk. No differences were seen between strategies during the in-trial period (5 years) or during post-trial follow-up (ten years). Therefore, the review showed no clinically adverse legacy effect on mortality or major CVD with delayed pharmacotherapy in middle-aged mildly-hypertensive subjects with low cardiovascular risk [54].

7. Legacy Effect after Lipid Control

Several clinical trials have assessed the legacy effects in different populations of patients with dyslipidaemia the main results of which are listed below. The specific data from each study are shown in Table 3.

Table 3. Studies assessing the legacy effect after lipid-lowering intervention.

	Lipid-Lowering Intervention			
	ALLHAT-LLT (*n* = 10.355/1.672) *	**WOSCOPS (*n* = 6.596/6.408) ***	**ASCOT-LLA (*n* = 4.605/4.432) ***	**ACCORD-Lipid (*n* = 940/765) ***
In-trial follow-up duration/Total follow-up duration	4.9 years/8–13 years	4.9 years/20 years	3.3 years/15.7 years	5 years/9.7 years
Patients characteristics	Men and women; mean age 66.4 years; hypertensive and at least one additional risk factor for CHD events	Men; mean age 55 years; high LDL cholesterol without CHD	Men and women; mean age 64 years; hypertension with at least three additional cardiovascular risk factors and total cholesterol ≤6.5 mmol/L	Men and women; mean age 61.4 years; long-standing T2DM with LDL between 60–180 mg/dL, HDL-C <55 mg/dL and triglycerides <750 mg/dL or <400 mg/dL on treatment
Type of intervention	Pravastatin vs. usual treatment	Pravastatin vs. placebo	Atorvastatin vs. placebo	Simvastatin + fenofibrate vs. simvastatin + placebo
Dietary intervention	Standard care	Standard care	Standard care	Standard care
In trial outcomes	No differences in all-cause mortality	Reduction in all-cause mortality, and death or hospitalisation for CHD	Reduction in nonfatal MI and fatal CHD	No benefits in cardiovascular outcomes and mortality; less major CHD events in hyperlipidaemic patients
Legacy effect	No	Yes	Yes	Yes
Effects in the post-trial follow-up	No reduction in CHD events and cardiovascular mortality	Reduction in all-cause and cardiovascular mortality, and lower cumulative hospitalisation event rates for any coronary event, MI, and heart failure in the pravastatin group	Reduction in cardiovascular mortality in the atorvastatin group	Reduction in all-cause and cardiovascular mortality, nonfatal MI, CHF and major CHD in the fenofibrate group; reduction in all-cause mortality in combined statin-fibrate arm

Abbreviations: CHD: coronary heart disease; CHF: congestive heart failure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MI: myocardial infarction; T2DM: type 2 diabetes mellitus. LLT: Lipid-lowering treatment; WOSCOPS: West of Scotland Coronary Prevention Study; LLA: lipid-lowering arm. * The number of randomised patients enrolled in the interventional phase and the cohort of post-trial follow-up, respectively.

The ALLHAT lipid-lowering trial (LLT) evaluated the impact of large, sustained cholesterol reductions in all-cause mortality in a cohort of hypertensive patients with at least one other cardiovascular risk factor. All-cause mortality did not differ significantly between the pravastatin and usual care treatment groups (RR 0.99, 95% CI, 0.89–1.11) at six years of follow-up. Similarly, no significant reductions in CHD events (RR 0.91, 95% CI, 0.79–1.04) were observed at the end of the follow-up period (Table 3) [55].

The West of Scotland Coronary Prevention Study (WOSCOPS) was a primary prevention trial performed in men (45 to 65 years old) with hypercholesterolemia who were randomised to pravastatin or placebo for five years. The 20-year follow-up study after the initial intervention identified a legacy benefit in the pravastatin group compared to placebo, with a significant reduction in all-cause mortality (HR 0.87, 95% CI, 0.80–0.94), attributable mainly to a 21% decrease in cardiovascular death (HR 0.79, 95% CI, 0.69–0.90), reduction in hospital admissions for any coronary event (18%), for MI (24%), and for heart failure (35%). The authors suggested that these benefits may be the result of reduced infarct size, prevention and regression of atherosclerotic changes, or pleiotropic effects of statins. On the other hand, there was no difference in non-cardiovascular or cancer death rates between groups (Table 3) [56].

The ASCOT lipid-lowering arm (LLA) is a substudy of the ASCOT study previously reported. ASCOT-LLA enrolled ASCOT study participants who had total cholesterol of 6.5 mmol/L or less and no previous lipid-lowering treatment. They were randomised to receive atorvastatin or placebo as primary prevention for CHD; lower nonfatal MI and fatal CHD was demonstrated in the atorvastatin group during the interventional period of the study. The legacy effect was studied for 16 years after initial randomisation and also showed a significant risk reduction in cardiovascular mortality (HR 0.85, 95% CI, 0.72–0.99) in those assigned to atorvastatin group. No differences in all-cause mortality or stroke were seen between the atorvastatin and placebo groups during the post-trial study period (Table 3) [52].

The ACCORD-Lipid study included a subgroup of ACCORD study participants. Briefly, T2DM patients were randomised to receive simvastatin plus fenofibrate vs. simvastatin plus placebo for five years; no evidence of a beneficial effect of statin-fibrate combined treatment compared to statins alone on cardiovascular outcomes and mortality was found. However, a beneficial reduction in major CHD (HR 0.65, 95% CI, 0.48–0.90) was observed in a subgroup of participants with dyslipidaemia. The extended post-trial follow-up study (ACCORDION) showed lower rates of all-cause mortality, cardiovascular mortality, nonfatal MI, CHF and major CHD in the simvastatin-fibrate group in the ten years following randomisation. Moreover, the trial period's combined statin-fibrate treatment arm conferred a beneficial legacy effect, which was observed in the post-trial follow-up on all-cause mortality (HR 0.65, 95% CI, 0.45–0.94). Fibrate therapy, offered as an add-on to statin therapy, may be beneficial for people with diabetes with hypertriglyceridemia and/or reduced HDL-C (Table 3) [57].

A recent meta-analysis, including eight placeboes vs. statin randomised clinical trials for primary and secondary cardiovascular prevention, evaluated the legacy effect during a mean post-trial follow-up ranging from 1.6 to 15.1 years. The results mostly showed a significant reduction in cardiovascular and all-cause mortality within the trial period, and less benefit in the post-trial period. The legacy effect was observed in relation to lower all-cause mortality but, appeared to have no effect on CVD mortality. Additionally, in a subgroup analysis, there appeared to be a greater legacy effect when statins were used for primary prevention compared to secondary prevention in CVD and all-cause mortality (HR 0.87 and 0.90, respectively), suggesting the importance of long-term prevention in these patients [58].

Another meta-analysis carried out by Hirakawa et al. (62) analysed the legacy effect in the post-trial follow up (mean duration six years) after an intervention period with antihypertensive and lipid-lowering treatments. The study demonstrated significant reductions in all-cause and cardiovascular mortality (about 9% and 12%, respectively) after discontinuation of antihypertensive and lipid-lowering treatment during the overall follow-up, although this effect was lower than that observed during the in-trial phase. Furthermore, progressive attenuation of post-trial benefits was

noted as the length of the post-trial observation increased. However, no clear differences between the effects of BP or lipid-lowering therapies were detected across trials studying different types of therapies or patient populations. These findings indicate that it is important to continue antihypertensive and lipid-lowering treatment in the long-term to provide optimal cardiovascular protection [59].

8. Legacy Effect after Multifactorial Intervention

The Action in Diabetes and Vascular Disease (ADVANCE) trial was performed in 11,140 T2DM patients ≥55 years old, with at least one additional risk factor for CVD. The trial proposed a double strategy to optimise glycaemic control (intensive glucose control or standard control) and BP control (perindopril-indapamide or placebo) for five years. During the intervention phase, there was a reduction in microvascular events (14%), primarily due to reduction in nephropathy incidence in the intensive glucose-control group, as well as a reduction in the relative risk of all-cause mortality (14%) and cardiovascular mortality (18%) in the BP control group. The six-year post-trial follow-up study found a significant reduction in all-cause mortality (HR 0.91, 95% CI, 0.84–0.99), and cardiovascular mortality (HR 0.88, 95% CI 0.77–0.99) in those patients originally treated with perindopril-indapamide compared to placebo. Although the confidence limits were wide, the results suggested that one death from any cause would be prevented for every 79 patients assigned to active therapy for five years. On the other hand, there was no evidence that the effects of the treatment could be inferred from initial BP levels or concomitant use of other treatments at baseline. However, intensive glucose control did not provide any long-term benefits during the intervention period nor after post-trial follow-up (Table 4) [60–62].

Table 4. Studies assessing the legacy effect after multifactorial intervention.

	Multifactorial Intervention	
	ADVANCE (*n* = 11.140/8.494) *	**Steno-2 (*n* = 160/96) ***
In-trial follow-up duration/Total follow-up duration	4.5 years (BP control) and 5-years (glycaemic control)/10 years	7.8 years/21.2 years
Patient characteristics	Men and women; mean age 66 years; T2DM with at least one additional risk factor for CVD and aged ≥55 years	Men and women; mean age 55.1 years; T2DM and microalbuminuria
Type of intervention	Combination of perindopril-indapamide vs. placebo. Intensive (gliclazide) vs. standard glucose control	Intensified multifactorial intervention (behavioural and pharmacological approaches) vs. standard therapy
Dietary intervention	Standard care	Dietary advice (total daily intake of fat ≤30% of total daily energy intake and less of 10% as saturated fat)
In-trial outcomes	Reduction in total and cardiovascular mortality in the intensive BP-lowering group. Reduction in nephropathy in the intensive-glycaemic control group	Reduction in the risk for progression to nephropathy, retinopathy and autonomic neuropathy, and decreased risk in cardiovascular complications and all-cause mortality combined in the intensive treatment group
Legacy effect	Yes (BP control)/No (glycaemic control)	Yes
Effects in the post-trial follow-up	Reduction in all-cause and cardiovascular mortality in the intensive BP-lowering group	Reduction in all-cause and cardiovascular mortality, and microvascular complications (nephropathy, retinopathy and autonomic neuropathy) in the intensive-therapy group

Abbreviations: BP: blood pressure; CVD: cardiovascular disease; T2DM: type 2 diabetes mellitus. * The number of randomised patients enrolled in the interventional phase and the cohort of post-trial follow-up, respectively.

The Steno-2 study recruited T2DM patients (*n* = 160; mean age 55 years) with microalbuminuria who were randomly assigned to standard treatment or intensified multifactorial intervention during a mean follow-up of 7.8 years. This intervention included simultaneous control of glucose levels, BP, lipid profile, and antiplatelet therapy through pharmacological and non-pharmacological intervention. The latter included dietary advice (total daily intake of fat ≤30% of total daily energy intake and less than 10% kcals as saturated fat), light to moderate exercise at least 30 min three to five times

a week, and smoking cessation. During the active intervention period, there was risk reduction for nephropathy progression (OR 0.27, 95% CI, 0.10–0.75), retinopathy progression (OR 0.45; 95% CI, 0.21–0.95), and autonomic neuropathy progression (OR 0.32, 95% CI, 0.12–0.78), as well as for cardiovascular complications and all-cause mortality combined (OR 0.45, 95% CI, 0.22–0.93) in the intensive treatment group. After a 21-year follow-up from randomisation, a significant reduction in all-cause mortality (45%), cardiovascular mortality (62%), macroalbuminuria (48%), retinopathy progression (33%), and autonomic neuropathy (41%) was demonstrated in the intensive treatment arm, suggesting a legacy effect from intensive multifactorial treatment compared to the standard approach. For patients in the intensive treatment group, death and time to first cardiovascular event was delayed by 7.9 and 8 years, respectively, compared to patients in the standard treatment group (Table 4) [63,64].

9. Discussion

Most studies evaluating the legacy effect on the primary or secondary prevention of CVD are clinical trials promoted and financed by the pharmaceutical industry in which an observational study has continued at the end of the intervention phase. As a consequence, the legacy effect has been assessed mainly after pharmacological intervention. Few studies have been carried out after a nutritional intervention, and most focused on animal and diabetic models.

Different clinical outcomes have been evaluated, including microvascular complications (retinopathy, neuropathy, nephropathy), major cardiovascular events (heart failure, MI, stroke, and ischemic gangrene) and mortality, as well as analytical parameters. The results obtained have been quite heterogeneous, probably due to differences in the baseline characteristics of the patients included, the type and duration of the interventions, and post-trial follow-up. In general terms, the legacy effect has been observed to be less intense than that achieved in the intervention phase and tends to diminish with longer follow-up. All the data suggest that this effect is greater and longer lasting in patients without known CVD undergoing intensive therapy for recent-onset diabetes, hypertension or dyslipidaemia.

A limited number of studies related to the legacy effect after nutritional intervention have been published to date. In studies performed with rats and mice, some authors have demonstrated that after CR (reduction of daily caloric intake by approximately 25%) or intermittent fasting, glycaemic control and insulin resistance could improve for several weeks. The evidence in humans is lacking, as health benefits of CR or intermittent fasting have been evaluated mainly in observational studies and a single clinical trial (CALERIE Study). The effect during the intervention period appears to be favourable but disappears shortly after the intervention ends. Therefore, CR or intermittent fasting has not been shown to generate a legacy effect in humans [30]. In addition, we would like to highlight safety concerns regarding intermittent fasting in patients with diabetes mellitus due to the higher risk of hypoglycemia. Data from the Oslo Cardiovascular study suggest that systematic advice on a healthy diet and smoking cessation for five years could be associated with a legacy effect translated into a reduced risk of cardiovascular mortality in the next 40 years. However, it is difficult to ensure that no confounding factors could have altered these results [32]. Currently, there are no data available on the possible legacy effect in other, more recent nutritional intervention studies, such as the PREDIMED (Prevención con Dieta Mediterránea) Study [65]. The limited scientific evidence published on the possible legacy effect in CVD prevention after a dietary intervention may be due to multiple factors, including: difficulty in maintaining high adherence to the proposed nutritional intervention, absence of specific biomarkers of dietary compliance and difficulty in having a real control group or blinding the interventions. In addition, these studies require a very long follow-up as well as a high economic cost that can be more difficult to finance if there is no financial support from public institutions. Due to all these limitations, there are few nutritional intervention studies that have carried out a long follow-up at the end of the intervention [66,67].

The first scientific evidence of the legacy effect in patients with diabetes comes from the DCCT and the UKPDS studies, in which an intensive glycaemic control compared to standard treatment resulted in a

significant reduction in cardiovascular mortality, nonfatal MI, stroke and nephropathy. These beneficial effects persisted for more than a decade after the intervention was finished [35,37]. Furthermore, the VADT trial provided the first evidence of the legacy effect in the reduction of a composite of major cardiovascular events (MI, stroke, CHF or amputation for ischemic gangrene) in patients with poorly controlled and long duration T2DM after ten years of randomisation. Notwithstanding, these significant cardiovascular outcomes disappeared over a longer follow-up period (15 years) [42,43]. On the other hand, the ADVANCE and ACCORD trials, which included long-standing T2DM patients, showed no legacy effect in CVD. Differences observed among these trials may be explained due to the fact that the DCCT and UKPDS studies recruited younger patients with new-onset diabetes without known CVD, in contrast to the characteristics of the patients included in the ADVANCE and ACCORD studies [40,62].

Several clinical trials have analysed the legacy effect after tight BP control with controversial results. On the one hand, the SHEP and the ROADMAP trials showed reductions in cardiovascular mortality, retinopathy and delayed onset of microalbuminuria, whereas the ASCOT study only demonstrated a small reduction in stroke mortality in the amlodipine-treatment group at the end of the entire follow-up [49,50,52]. On the contrary, the HDS and the ALLHAT studies did not provide clear evidence of a legacy effect, suggesting that this beneficial effect in patients with higher cardiovascular risk and probable subclinical CVD is unlikely to be achieved once in the post-trial phase, when BP is less strictly controlled. In addition, the benefits of antihypertensive treatment on major cardiovascular outcomes usually appear shortly after treatment implementation, and are attenuated when BP differences between groups are lost [47,48]. Therefore, based on clinical trials conducted, it appears that the legacy effect does not exist or seems to be mild and transient after tight antihypertensive regimens.

Different lipid-lowering trials, including ASCOT, WOSCOPS and ACCORD lipid studies, have also observed a legacy effect, resulting in a reduction in all-cause mortality and CHD mortality for 10–20 years after ending the interventional phase (statin or fibrates treatment), whereas the ALLHAT-LLT study did not provide evidence of a legacy effect after intensive lipid-lowering treatment [52,55–57]. It should be noted that in those trials in which a legacy effect was observed, the statin, a drug with pleiotropic effects, was compared against placebo. Moreover, the observation that five years of statin therapy led to a lower long-term risk of all-cause and CVD mortality raises the question of whether treatment with statins for 5–10 years would be sufficiently beneficial, while limiting lifetime exposure to the drug. However, there are still concerns about whether this therapeutic strategy could be effective in any patient, or in select populations only.

Until now, the legacy effect after multifactorial intervention has only been observed in the Steno-2 study, which included pharmacological and non-pharmacological measures. Indeed, lower mortality, CVD incidence and microvascular complications were detected in the intensive treatment arm of the trial compared to the standard treatment group. Surprisingly, the results were obtained in a sample of only 160 patients with intermediate cardiovascular risk but without CVD at baseline. It has been suggested that the positive long-term cardiovascular effects must be the result of a synergistic effect of the multifactorial intervention on cardiovascular risk factors [64]. Although these results are impressive, they have not been reproduced in subsequent studies.

The knowledge of pathophysiological mechanisms underlying the legacy effect adds plausibility to its existence. The best-known mechanisms are those related to the deleterious effects of hyperglycaemia through the development of metabolic memory induced during the first years of diabetes onset, which cannot be reversed with better glycaemic control. Thus, early interventions against hyperglycaemia could reduce ROS production and oxidative stress in the mitochondria of endothelial cells, decrease AGE formation and RAGE expression, and therefore, prevent activation of inflammatory processes and epigenetic changes in the arterial walls in the long term [4,5,15–17,20,21]. Likewise, dysregulation of RAAS may be involved in vascular complication development, through increased oxidative stress and AGE formation. However, these mechanisms are not yet fully understood [19].

This review has several strengths and limitations. Firstly, one strength involves the inclusion of well-designed multicentre prospective clinical trials with large participant samples during the interventional phase. Secondly, most trials have a long post-interventional follow-up (6–20 years), which is enough time to detect differences between groups. Likewise, the study of different types of cardiovascular risk factors (diabetes, hypertension or dyslipidaemia), as well as variable evolution time and treatment objectives (primary or secondary prevention of CVD) may allow us to identify in which studies the legacy effect may be more or less relevant. However, some limitations should be taken into account. First, loss of patients during post-interventional follow-up can reach 25–30% of subjects included in the trial (attrition bias), therefore data collection during this period may not have been as exhaustive as in the intervention period. The publication of only those trials with positive results (publication bias) and the absence of blindness in several trials (co-intervention bias) should also be considered. Furthermore, the inclusion of post hoc analysis together with the detection of the legacy effect is influenced by different confounding factors (recommended therapeutic targets, adherence to treatment, global cardiovascular risk), which may lead to a questionable interpretation of the results. Finally, another limitation that must be taken into account is the fact that few non-pharmacological studies (i.e., dietary intervention studies) have been carried out. Although dietary recommendations are included in most studies, they are usually quite generic, and it is also difficult to know the degree of adherence to them.

In conclusion, there is sufficient data to suggest the existence of a legacy effect after intensive intervention on cardiovascular risk factors in subjects with moderate-high vascular risk. However, this effect is not equivalent for all risk factors and could be influenced by patient characteristics, disease duration and the type of intervention performed. Currently, the available evidence suggests that the legacy effect would be greater in subjects with moderate-high cardiovascular risk but without known CVD, especially in patients with recent-onset diabetes. However, we should not withdraw any treatment to prevent CVD in these individuals as the level of available evidence on the legacy effect is low to moderate. Further investigation should be promoted to determine whether there is a legacy effect associated with nutritional interventions.

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Review

Very Long Chain Marine *n*-3 Polyunsaturated Fatty Acids in Atherothrombotic Heart Disease. A Brief Review, with a Focus on Metabolic Effects

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Abstract: The global burden of atherothrombotic heart disease should be considered as a life-style disorder where differences in dietary habits and related risk factors like limited physical activity and adiposity together play important roles. Related metabolic changes have been scientifically elucidated in recent decades, and the role of the very-long-chain marine fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been much focused on, especially their possible effects on processes like inflammation and thrombosis. In the present brief review of related metabolic mechanisms, the effects of these fatty acids in a clinical setting have been referred to, including some of the authors' work on this topic. The main focus is the divergent results in the field and the important differences between the study population, the type of supplements and fresh marine sources, the proportion of EPA versus DHA dosages, and the duration of supplementation in clinical trials. We conclude that daily intake of at least 1 g of EPA + DHA may improve a dysmetabolic state in the population. The potential to reduce the risk and progression of atherothrombotic heart disease is still a matter of debate.

Keywords: omega-3 fatty acids; metabolism; inflammation; atherothrombosis; clinical trials

1. Background

Atherothrombotic heart disease is a dominating cause of morbidity and mortality worldwide [1,2]. Atherothrombotic heart disease comprises the continuous process of atherosclerosis, leading to narrowing of the coronary arteries and possibly the clinical manifestation angina pectoris, and the acute thrombotic occlusion of the artery as a basis for the life-threatening myocardial infarction [3,4]. Lifestyle factors like dietary habits have been highlighted as risk factors for atherothrombosis [5–7]. Preventive approaches like achievable dietary changes are obviously of major importance both for health and economic reasons. The importance of increased intake of very-long-chain *n*-3 polyunsaturated fatty acids (VLCM *n*-3 PUFAs), especially from marine sources, has been discussed over the last 50 years, back to the observations on Greenland Eskimos by the Danish scientists Dyerberg and Bang [8,9]. They found that the occurrence of coronary heart disease was very low among the Eskimos compared to the regular Danish population, and they attributed this observation to their special diet, which was composed mainly of seal and whale and was extremely rich in marine *n*-3 FAs. Later, a series of mechanisms for the potentially beneficial effects of such VLCM *n*-3 PUFA were published [10–15].

Relevant biologically active families of polyunsaturated FAs are the *n*-6 and *n*-3 FAs that contribute to several important metabolic pathways. However, as mammals lack enzymes to insert double bonds in the *n*-6 and *n*-3 position, linoleic acid (LA) (18:2 *n*-6) and alfa-linolenic acid (ALA) (18:3 *n*-3) are

essential nutrients. In addition, the elongation and desaturation of these FAs to the metabolically important VLCM PUFAs are limited, and the *n*-3 and *n*-6 series are competing for the same enzymes, the elongases, and the desaturases (Figure 1). The metabolic key FAs in the two series are arachidonic acid (AA) (20:4 *n*-6) in the *n*-6 series and eicosapentaenoic acid (EPA) (20:5 *n*-3) and docosahexaenoic acid (DHA) (22:6 *n*-3) in the *n*-3 series [12,13,15].

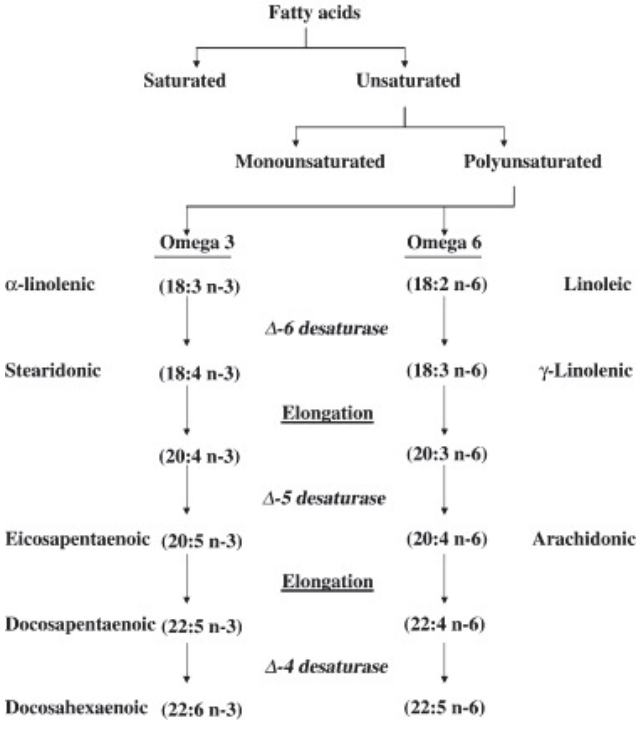

Figure 1. Metabolic pathway of omega-6 and omega-3 fatty acid synthesis. Copied from Siddiqui RA, Harvey KA, Zaloga GP. *J. Nutr. Biochem.* **2008**, *19*, 417–437 [10], with permission.

In regular Western diet today, the content of LA (from green plants and red meat) is much higher than that of ALA. Thus, the competition for elongases and desaturases favors the synthesis of the *n*-6 series, i.e., mainly AA, which is metabolized into mainly pro-inflammatory and platelet activating derivatives. Therefore, the synthesis of the very-long-chain EPA and DHA are minimal, especially when the diet is relatively low in ALA [16,17]. Derivatives from EPA/DHA seem to be rather neutral with respect to inflammation and platelet activation, but contribute importantly to the resolution of inflammation by being so-called resolvins [13,18].

In recent decades, in addition to epidemiologic and registry studies, a series of observational and randomized clinical trials on the possible clinical effect of intake (dietary or as supplements) of VLCM *n*-3 PUFA have been conducted [13–15]. Additionally, the effects on in vivo VLCM *n*-3 PUFA in the circulation, adipose tissue, and erythrocyte membrane have been extensively studied [19–21]. The results are divergent, mainly depending on (i) the population studied, i.e., baseline health status and risk factors, primary versus secondary prevention, and background dietary intake of *n*-3 and *n*-6 FAs; (ii) the type of supplementation (fatty fish, cod liver oil, seal oil, or capsules); (iii) duration of supplementation; (iv) dosage of supplementation; (v) proportion of EPA and DHA; and (vi) the quality of capsule content.

As in all therapeutic trials, compliance with the investigational principle is relevant to assess the validity of the results. Dietary questionnaires are often used to characterize the diet, but are generally inadequate for scientific purposes, although validated scoring systems are available. In studies using supplementary capsules, "pill count" is often used as a measure of compliance. However, this approach has important scientific limitations relating to logistics and patient reliability. In addition, supplementary capsules are prone to peroxidation of the unsaturated FAs, although often add anti-oxidants. Thus, in vivo measurements of the relevant FAs in the circulation, erythrocyte membrane, and adipose tissue are considered to be the scientifically most reliable methods. This strategy also allows for the estimation of relevant indexes (EPA + DHA/total FAs % and *n*-6/*n*-3 ratios) [21–23].

It should be emphasized that the unsaturated FAs are prone to the peroxidation and saturation of their double bonds, potentially reducing their beneficial effects in atherosclerotic cardiovascular disease states [24,25]. This is often the case with commercial supplements of VLCM *n*-3 PUFAs if not enriched with antioxidants like vitamin E. Fresh marine fish products do not experience the same risk of peroxidation.

2. VLCM *n*-3 FA, Metabolic Disorders and Atherothrombotic Heart Disease

The metabolic syndrome is composed of the most important metabolic disorders related to atherothrombotic heart disease. It describes a cluster of risk factors for cardiovascular disease states (CVD) and diabetes type 2, including abdominal obesity (waist circumference ≥ 102 (men)/88 cm (women)), elevated fasting blood glucose (≥5.6 mmol/L), hypertension (blood pressure ≥130/85 or on treatment), elevated triglycerides (≥1.7 mmol/L), and low levels of HDL cholesterol (<1.0 (men)/1.3 (women) mmol/L), according to the scientific statement from the American Heart Association and National Heart, Lung, and Blood Institute [26].

Among these risk factors, triglycerides and hypertension have been given most attention as relevant targets for potential effects of the VLCM *n*-3 PUFA. Regarding triglycerides, fasting samples are most frequently used, although non-fasting values have lately been used in epidemiological studies [27]. Triglycerides have been repeatedly shown to be predictive of future CVD, and their importance in risk prediction has increased in parallel with the increased prevalence of the metabolic syndrome in the Western world. Already in 1992, this effect was demonstrated in two large population studies from Germany and Finland [28,29], and later was documented in several populations with long-term follow-up, like the PROCAM study [30]; the Oslo Study [31]; and, more recently, the Copenhagen Study [27].

Furthermore, the triglyceride-lowering effect of the VLCM *n*-3 PUFAs has been observed in numerous clinical trials and is considered a key pathway in preventing CVD. The mechanism by which these VLCM *n*-3 PUFAs reduce triglycerides is probably by reduced hepatic synthesis and secretion [32], and reduction of triglycerides has often been used as a measure of compliance in clinical trials. The dose-relationship is well known and is evident in the various clinical trials using a range of VLCM *n*-3 PUFA doses. In the GISSI-Prevenzione trial [33] using a low dose (0.85 g/day), the reduction in triglycerides was only 3.4% over 5.5 years, whereas in the CART study [34] with a very high dose of 5.1 g/day, the reduction over 1 year was 27%. In the SHOT [35] and DOIT [36] studies, relatively high doses of 3.4 g/day and 2.4 g/day, respectively, were used, resulting in 19 and 16% triglyceride reduction, respectively. In the recent REDUCE-IT study [37], the reduction with 4 g/day of icosapent ethyl (an ethyl-EPA that is metabolized to EPA after ingestion) over 4.9 years was 18%. In the latter study, the reduction of cardiovascular risk could not be explained solely by the reduction in triglycerides, suggesting other cardio-protective mechanisms from high-dose EPA [38]. In the 2019 European Society of Cardiology/European Atherosclerosis Society guidelines VLCM *n*-3 PUFA has a IIa recommendation for lowering triglycerides [39]. High doses of the VLCM *n*-3 PUFA are recommended for very high levels of triglycerides, mainly to avoid pancreatitis [40]. Whether even higher doses, e.g., 8–12 g/day

would have even better longstanding effects is unclear, but at these doses bowel dysfunction may be a challenge.

In lipidology, there is a well-known inverse relationship between triglycerides and HDL-cholesterol, although this may differ between subtypes of HDL-cholesterol. Thus, with high levels of triglycerides low levels of HDL-cholesterol are regularly found. In studies on supplementation with VLCM *n*-3 PUFAs, the reduction in triglycerides is regularly accompanied by a moderate increase in HDL-cholesterol. A more specific effect on the synthesis or secretion of the latter has, however, not been clearly elucidated.

With respect to hypertension, the VLCM *n*-3 PUFAs have been shown to reduce both the systolic and diastolic blood pressure. Already in 1990, Bønaa et al. [41] could demonstrate in a randomized, control trial in 156 hypertensive men and women, a significant, although moderate, reduction of both SBP and DBP after six weeks supplementation with 5.1 g/day, and the effect was related to the measured increase in plasma *n*-3 FAs. Similar effects have later been demonstrated in larger meta-analyses [20,42].

In the metabolic syndrome, hyperglycemia and insulin resistance are linked to abdominal adiposity and a pro-inflammatory state in the adipose tissue (and in general). In murine and human studies investigating supplementation of VLCM *n*-3 PUFAs, a tendency to change from a pro-inflammatory state into an anti-inflammatory state of the adipose tissue has been shown [43,44]. Thus, a transition of macrophages from the pro-inflammatory M1-type to the anti- inflammatory M2-type in response to VLCM *n*-3 PUFAs has been described [45]. In vitro cellular studies have demonstrated that these metabolic effects are partly mediated by peroxisome–proliferator–activator receptor (PPAR) transcription factors, typically with two receptor subtypes, PPARα and PPARγ. Whereas PPARα is also thought to mediate the beneficial effect of VLCM *n*-3 PUFAs on hepatic insulin sensitivity, PPARγ mediates the effect on adiponectin upregulation [44]. Both subtypes are also known to counteract the activation of the key proinflammatory transcription factor NFkB [12,13,15,44,46] (Figure 2).

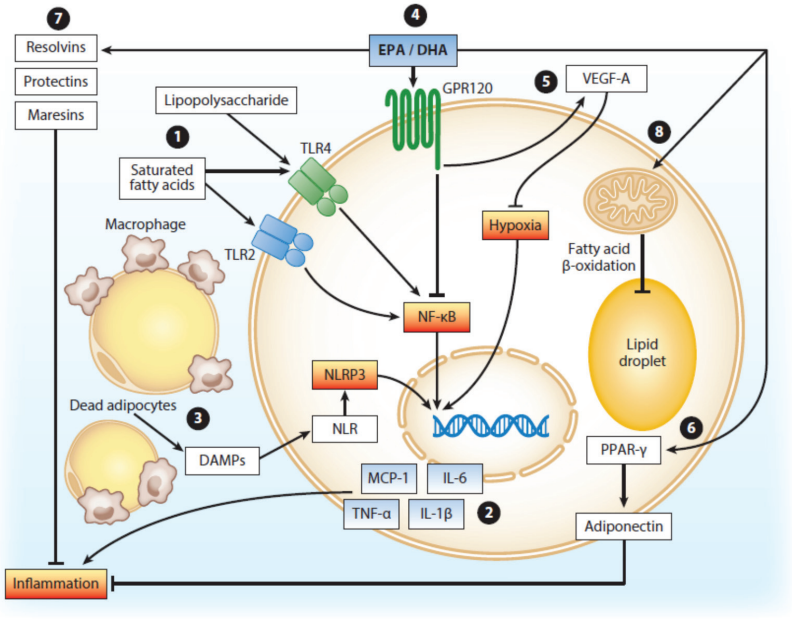

Figure 2. Modulation of white adipose tissue function by omega-3 polyunsaturated fatty acids (ω-3 PUFAs). Copied from Kalupahana NS et al., with permission. *Annu. Rev. Nutr.* **2020** Jun 16. doi:10.1146/annurev-nutr-122319-034142 [44].

Another rapidly evolving area has been the role of micro-RNAs (miRNAs) in the inflammatory balance in white adipose tissue, and their possible anti-inflammatory role, also related to the presence of insulin resistance [43]. Protective effects of VLCM *n*-3 PUFAs has also been linked to certain miRNAs, and the importance of the ratio between *n*-6 and *n*-3 FAs in the diet, as well as in circulation and adipose tissue, has been emphasized [43].

Regarding adiposity per se, especially abdominal adiposity, the influence of VLCM *n*-3 PUFAs and the *n*-6/*n*-3 ratio has mainly been focused on the pro-inflammatory state and insulin resistance. It emerges from both animal and human studies that the modern Western diet with increased *n*-6/*n*-3 ratio is associated with increased incidence of adiposity, and increased pro-inflammatory activity in the adipose tissue, increased insulin resistance, and increased risk of type 2 diabetes. Results from studies with supplementation of VLCM *n*-3 PUFAs in the diet or as purified supplements indicate that reducing the *n*-6/*n*-3 ratio counteracts adiposity and normalizes the dysmetabolic state in the metabolic syndrome [43,46–49]. This effect may be linked to the anti-inflammatory properties of VLCM *n*-3 PUFAs by their role in resolution of inflammation (Figure 3).

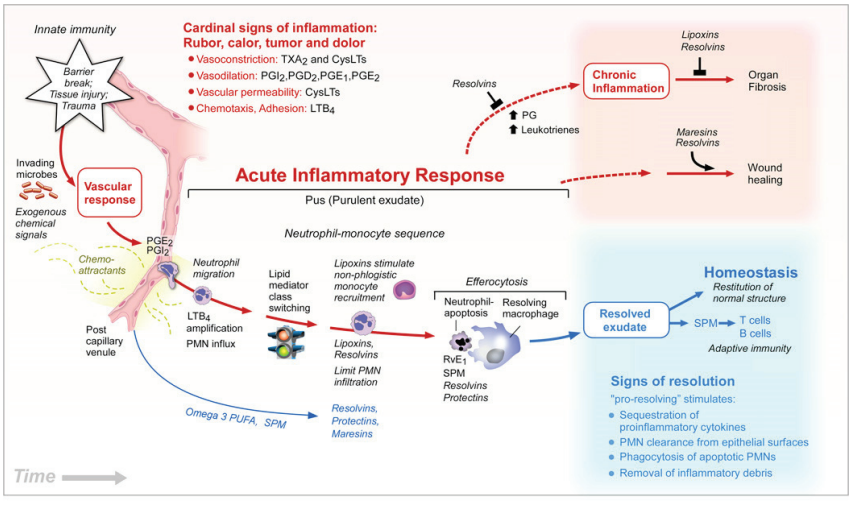

Figure 3. Lipid mediators in the acute inflammatory response, resolution, and other outcomes. Copied from Serhan CN, *Nature* **2014**, *510*, 92–101 [18], with permission.

Additionally, the possible importance of polymorphisms in the regulation of fatty acids desaturases (FADS) for the *n*-6/*n*-3 ratio has been focused on [50]. Specific clustering of these polymorphisms on chromosome 11 seems further to be related to sex and compliance with a Mediterranean diet.

3. VLCM *n*-3 PUFAs Role in Cardiovascular Risk Assessement

Since the Diet and Reinfarction Trial (DART) in 1989 found a 29% reduction in 2-year mortality associated with increased fish intake (fatty fish twice per week) in approximately 2000 post-MI patients [51], several randomized clinical trials have investigated the effect of VLCM *n*-3 PUFA in reducing cardiovascular risk. Most studies have been performed with use of supplements. The GISSI-Prevenzione trial, also a secondary prophylaxis study, demonstrated in 1999 a significant reduction in all-cause mortality (21%) and 45% reduction in sudden cardiac death in ~11,000 patients randomized to 0.85 g EPA + DHA/day versus placebo [33]. The first large RCT testing EPA alone was the Japan EPA Lipid Intervention Study (JELIS), randomizing more than 18,000 individuals already treated with statins, to 1.8 g EPA/day versus placebo for 5 years [52]. They found a 19% reduction in major coronary events, but no significant reduction in CV death. Interestingly, patients with

hypertriglyceridemia were identified as super-responders, with a 53% reduction in major coronary events. In the last decade, several studies have investigated low-dose EPA + DHA in secondary prevention without any convincing cardiovascular benefit [53–56].

In 2018, three much anticipated RCTs on primary prevention were published. The Vitamin D and Omega-3 Trial (VITAL) randomized almost 26,000 healthy participants to 1 g EPA + DHA/day and followed them for 5 years [57]. They did not find any significant reduction in the composite endpoint of AMI, stroke, and CV death. The ASCEND (A Cardiovascular Study of Events in Diabetes) Study included more than 15,000 patients with diabetes mellitus and randomized them to ~1 g of EPA + DHA/day versus placebo [58]. Also in this study, there was no significant reduction in cardiovascular events over 7 years of follow-up. The third study was markedly different in design and treatment. The Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial (REDUCE-IT) tested 4 g/day of icosapent ethyl versus placebo in 8179 statin-treated adults with hypertriglyceridemia [37]. A striking 25% reduction in the primary composite endpoint of CV death, nonfatal MI, stroke, coronary revascularization, and unstable angina was observed after 5 years.

A separate mechanistic RCT (EVAPORATE) used serial coronary CT scans to evaluate changes in coronary plaque burden in REDUCE-IT-like patients with 4 g/day of icosapent ethyl [59]. This study showed significant reduction in the primary endpoint of changes in low-attenuating plaque volume (−17% vs. +109% in the placebo group), and a significant reduction also in fibro-fatty plaques (−34% vs. +32%) but not in calcified plaques (−1% vs. +15%) after 18 months. Based on these studies, and category A science Advisory from the American Heart Association [40], the Food and Drug Administration (FDA) in 2019 approved icosapent ethyl as an adjunctive therapy to reduce the risk of CV events in patients with hypertriglyceridemia and high risk of CVD.

During the last 30 years, we have studied a variety of effects of the VLCM *n*-3 PUFAs in randomized clinical trials as well as by use of extensive biobanks. Back in the early 1990s, the SHOT study [35] was performed to study whether supplementation with VLCM *n*-3 PUFAs (3.4 g/day of highly concentrated EPA + DHA) would positively influence the occlusion rate of coronary artery bypass grafts (CABG) compared to controls (n = 610). Vein graft occlusion rates after one year, assessed by angiography, were significantly lower (27%) in the VLCM *n*-3 PUFA group compared to controls (33%). Moreover, a significant trend to lower vein graft occlusion rate with increasing relative change in serum *n*-3 PUFA levels during the study period was observed. Simultaneously, serum levels of EPA increased from 38 to 92 (mg/L), and serum levels of DHA from 113 to 129 (mg/L), in the active treatment group. To the best of our knowledge, similar randomized trials have not been conducted.

In the CART study [34], performed later in the 1990s, supplementation with VLCM *n*-3 PUFAs was given daily for six months with the hypothesis to reduce the frequency of restenosis after percutaneous transluminal coronary angioplasty (PTCA). Patients were randomized to either 5.1 g/day of highly concentrated EPA + DHA or corn oil as placebo, starting two weeks prior to PTCA to allow for the FAs to be incorporated in the cell membranes before the procedure. Despite that, serum levels of EPA and DHA increased from 43 to 101 mg/L and 112 to 132 mg/L, respectively, in the VLCM *n*-3 PUFA group, no effects on the restenosis frequency were obtained (40.6% in the active treatment group vs. 35.4% in the placebo group). This is line with results from other PTCA studies conducted in the same time period using this very high dose of supplementation [60,61]. Sub-studies, however, revealed that whereas this high dose of VLCM *n*-3 PUFAs was followed by reduction in pro-thrombotic markers, markers of inflammation were increased, possibly related to lipid peroxidation visualized by reduction in vitamin E and increase in thiobarbituric acid-reactive substances [62].

The possible pro-oxidative properties of high doses of VLCM *n*-3 PUFAs have been discussed with conflicting conclusions [24,25,63,64]. This topic has mainly been explored in experimental studies, and limited in the context of patients with cardiovascular disease. In the OVITES study, 6 weeks' worth of supplementation with either low (0.85 g/day) or high (5.1 g/day) dose of VLCM *n*-3 PUFA, or the latter amount + vitamin E 400 mg/day, was explored in high risk individuals. In the low FA group, no changes in inflammatory or peroxidation markers were observed, whereas in the high FA group

alone significantly increased inflammation and peroxidation were noted, without any change when vitamin E was added [65]. Thus, the high dose of VLCM *n*-3 PUFA may induce lipid peroxidation in individuals at risk of atherosclerosis with a subsequent proinflammatory state, not counteracted by vitamin E as an antioxidant, probably because peroxidation mainly occurs at the cellular level. Nevertheless, whether higher doses have a better longstanding effect is still unclear, and is probably dependent on the population. It should be noted that daily dietary amounts in Greenland Eskimos were described as being about 10 g and even more [66]; however, this population at that time is not comparable with the general Western world population today.

In the DOIT study [36,67], the possible modulating effect of 3-year diet counselling was compared to that of supplementation with VLCM *n*-3 PUFAs given as capsules, on the progression of atherosclerosis assessed as structural measures of carotid IMT (cIMT), plaques score, and vascular function, as well as on clinical end-points. Such comparisons in clinical studies on atherosclerosis had hitherto not been extensively studied. Men at high risk for CVD were randomized in a factorial design to dietary counselling and/or VLCM *n*-3 PUFA capsules (2.4 g VLCM *n*-3 FA/day) in a placebo-controlled fashion. The dietary advices were to increase the use of vegetable oils, vegetables, fruits, and fish, and to decrease the use of meat and fat from animal sources. Reduced progression in cIMT was observed in groups with dietary counselling, whereas vascular function was significantly improved in the VLCM *n*-3 PUFA groups and significantly correlated to changes in serum EPA concentrations. When applying the so-called Plasma Index (PI) (EPA+DHA/total FAs) [22] to the *n*-3 PUFA groups, baseline levels of total FA were 5.5 g/L, PI 2.2% with EPA and 3.3% with DHA, and after 36 months total FA was 5.0 g/L. PI with EPA was increased to 4.5% and with DHA to 4.3%. Although the study was not geared toward clinical endpoints, a 50% reduction of all-cause mortality in patients randomized to VLCM *n*-3 PUFA supplementation, compared with placebo, was obtained [67].

Vegetable oils are increasingly used in fish farming industry due to shortness of marine resources; however, the effects on human health of this feeding change are largely unknown. We studied the effects of dietary intake of Atlantic salmon, fed on different fatty acid sources, on the lipid profile, vascular inflammation, and peroxidation in patients with stable coronary heart disease, in a double-blind, randomized dietary intervention study ("Fiord-to-Table") [68,69]. Patients were randomized into three groups consuming approximately 700 g per week for 6 weeks of differently fed Atlantic salmon (fed (i) 100% South American fish oil, (ii) 50% South American fish oil/50% rapeseed oil, and (iii) 100% rapeseed oil, respectively). Significant differences between the groups in serum levels of total VLCM *n*-3 PUFA and the *n*-3/*n*-6 FA ratio were demonstrated, and interestingly, the fatty acid composition of the fish feed/pellets and fish fillets was highly mirrored in the serum FA profile of the patients after the intervention: "The patient's serum fatty acid profile became what the salmon was fed" (Figure 4). Additionally, significant reduction in serum triglycerides and inflammatory markers were obtained in patients receiving the 100% South American fish oil.

Possible changes in dietary habits during the study period when ingestion of 700 g salmon per week was also examined [69]. In the total population, significant changes were observed for fatty fish (10.2–75.9 g/day), lean fish (18.6–0.0 g/day), processed fish (16.4–0.8 g/day), processed meat (23.1–15.8 g/day), pork (11.1–7.8 g/day), and meat stew (30.2–22.7 g/day). Thus, a healthier diet was obtained, and the beneficial results of vascular inflammation with 100% South American fish oil were conferred by the difference in feed of the farmed salmon. To the best of our knowledge, similar studies have not been conducted since.

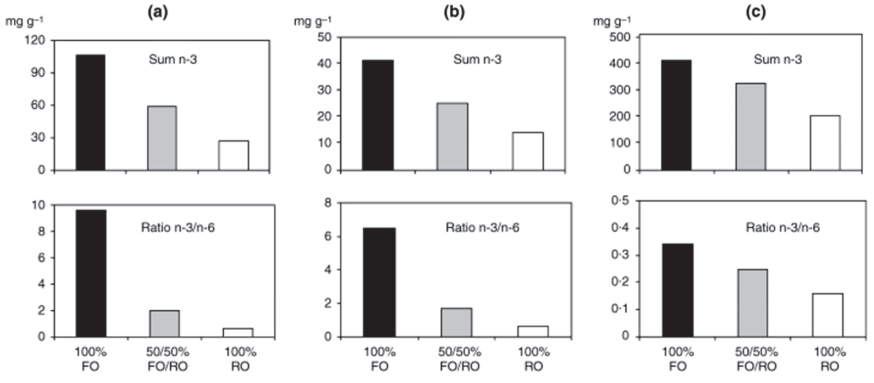

Figure 4. Patterns of the sum of *n*-3 FA and the ratio *n*-3/*n*-6 FA in (**a**) the three different feeds, (**b**) corresponding salmon fillets, and (**c**) serum fatty acids in patients, after the 6-week dietary intervention. FO: fish oil; RO: rapeseed oil (Seierstad SL et al. [67]).

In this context, the quality of farmed salmon on the market can be discussed, as can the toxicity of farmed fish. In this "Fiord-to-Table"study, the latter was explored, showing levels of inorganic contaminants in plasma to be only slightly changed over the study period [70].

The ongoing OMEMI Trial (NCT01841944NC) [71] is a prospective, randomized, placebo-controlled, double-blind multicenter trial, aimed to investigate the effects of supplementation with 1.8 g/day of VLCM *n*-3 PUFAs (Pikasol®) on cardiovascular morbidity and mortality during a follow-up period of 2 years in an elderly population (70–82 years) who have experienced an AMI. Compliance is secured by measurement of FAs in serum phospholipids. Our hypothesis is that supplementation of VLCM *n*-3 PUFAs will reduce the risk for cardiovascular events. All patients are included and follow-up is finalized. The results will be presented at the end of 2020.

4. Summary

The global burden of atherothrombotic heart disease should be considered a life-style disorder where differences in dietary habits and related risk factors like limited physical activity and adiposity together play important roles. Related metabolic changes have been scientifically elucidated in recent decades, and the role of VLCM FAs EPA and DHA has gained increased interest. Their possible effects on pathophysiological processes like inflammation and thrombosis have been highlighted.

In the present brief review, we have mainly focused on metabolic mechanisms in atherothrombotic heart disease, and the effects of these FAs in the clinical setting. The main focus has been on the divergent results in the field and the important differences in study population, supplements versus fresh marine sources, the proportion of EPA versus DHA, and the dosages and duration of supplementation.

We conclude that daily intake of at least 1 g of VLCM FAs may improve the dysmetabolic state. The potential to reduce the risk and progression of atherothrombotic heart disease is, however, still a matter of debate.

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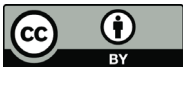

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Animal Protein Intake Is Positively Associated with Metabolic Syndrome Risk Factors in Middle-Aged Korean Men

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Abstract: Few studies have examined the relationship of protein intake by food source with metabolic syndrome in Korean adults, even though animal food intake has increased. This study examined the association between plant and animal protein intake and metabolic syndrome among middle-aged Korean adults. A total of 13,485 subjects aged 30–64 years were selected from the 2013–2018 Korea National Health and Nutrition Examination Survey. Protein intake was assessed using 24-h dietary recall data and divided into quintiles. Men had a higher percentage of energy intake from animal protein (7.4%) than plant protein (6.9%). Men in the highest quintile group of animal protein intake had a higher prevalence of abdominal obesity (OR: 1.30, 95% CI: 1.00–1.70), reduced high-density lipoprotein cholesterol (HDL-C) (OR: 1.43, 95% CI: 1.07–1.90), and elevated fasting glucose (OR: 1.32, 95% CI: 1.01–1.74), after adjusting for covariates. Furthermore, stronger associations of animal protein intake with abdominal obesity were shown in men who consumed less than estimated energy requirements (OR: 1.60, 95% CI: 1.11–2.31). Plant protein intake was negatively associated with increased blood pressure in men. Neither animal nor plant protein intakes were significantly associated with any of the metabolic syndrome risk factors in women. The results imply that lower animal protein intake may be a beneficial factor for metabolic syndrome management in middle-aged Korean men.

Keywords: abdominal obesity; animal protein; protein intake; metabolic syndrome

1. Introduction

Metabolic syndrome, defined as comorbidities of metabolic abnormalities including dyslipidemia, high blood pressure, glucose intolerance, and abdominal obesity, is a public health concern due to its link to increased risks of diabetes, cardiovascular disease, and all-cause mortality [1–3]. Various strategies including lifestyle modification such as a healthy diet are recommended to manage metabolic syndrome and prevent other chronic diseases [4]. Macronutrient composition is a major factor in determining a healthy diet which is known to be beneficial for weight loss and metabolic risk factors [5–7]. It has also been reported that dietary protein content is an important factor affecting the glycemic response related to glucose metabolism [8]; however, the role of dietary protein in the risk of metabolic disease remains inconclusive.

Diets high in protein have proved helpful for weight loss, with increased satiety and decreased energy intake, and improved cardiovascular markers including lipid profiles and blood pressure [9,10]. However, a high protein diet is also reported to induce metabolic disorders. High protein intake has the potential to stimulate hyperinsulinemia [11]. In addition, several studies have found that long-term high protein intake is associated with an increased risk of weight gain, type 2 diabetes, and overall mortality [12,13]. These disparate associations between protein intake and metabolic diseases seem to depend on the source of protein intake; for example, animal protein intake was positively associated with type 2 diabetes in a meta-analysis [14], whereas plant protein intake was negatively associated with high blood pressure in a prospective cohort study [15].

The reason for the disparate association according to protein sources can be explained by the quality of the protein and varied intake of other macronutrients. Protein quality is determined by its composition of indispensable and dispensable amino acids, which vary according to meat, and non-meat or plant-based foods. The amino acid composition may affect the digestibility of the protein [16], which can be linked to specific biological mechanisms. In addition, increasing protein intake alters the intake of other nutrients such as refined carbohydrates and saturated fat, depending on which protein sources are mainly consumed, since protein is derived from various food sources. Therefore, the intake of a particular protein source and the subsequently replaced nutrients are the major factors that explain the effects of increased protein intake on metabolism [17].

Traditional Korean diets are characterized by a high composition of various plant foods [18,19]. However, Korean diets have become westernized, particularly in the younger population [20], and the consumption of animal food products has increased [21–23]. The relationship between protein intake by food source and metabolic diseases among Koreans has been examined, but limited to the elderly population [24–26], while there are many studies including younger and older adults from other countries [14]. A Western diet has been linked to a high risk of metabolic diseases [20,27,28], and the clinical outcomes among younger adults are different from the elderly, since their dietary intakes including protein intake differ [29].

Evidence regarding the effect of protein intake by food source on the risk of metabolic syndrome for adults is required for metabolic syndrome management among Korean. To elucidate this issue, we examined the association of plant and animal protein intake and metabolic syndrome among middle-aged Korean adults using nationally representative population-based survey data.

2. Materials and Methods

2.1. Study Subjects

Data were obtained from the 2013–2018 Korea National Health and Nutrition Examination Survey (KNHANES), which is conducted annually by the Korea Centers for Disease Control and Prevention (KCDC). KNHANES consists of three examinations: a health interview, health examination, and nutrition survey. The health interview collects information on socio-demographic and health behavior variables, the health examination conducts anthropometric and blood parameter measurements, and the nutrition survey captures diet-related data, such as food and nutrient intake, food frequency, and dietary behavior [30]. All examinations are performed by trained interviewers or medical personnel with the participants' written consent. The protocols for data collection were approved by the Institutional Review Board of the KCDC (2013-07CON-03-4C, 2013-12EXP-03-5C, and 2018-01-03-P-A).

Of 23,355 adults aged 30–64 years, subjects were excluded if their energy intake was implausible (<500 or >5000 kcal/day, n = 3347), if they had been diagnosed with or were being treated for hypertension, hyperlipidemia, or diabetes (n = 4633), if they were pregnant or lactating (n = 247), and if they had missing data on major variables, including 24-h dietary recall and metabolic syndrome parameters (n = 1643). A final total of 13,485 subjects were included (men: 5318 men; women: 8167).

2.2. Dietary Assessment

Dietary data, such as nutrients and food intake, were obtained using a 24-h dietary recall method by face-to-face interviews at the participant's house. Protein intake was divided into plant or animal protein intake based on food sources. The plant protein groups included refined grains, other grains, legume and legume products, vegetables, fruits, condiments, and nuts and seeds. Refined grains included white rice, noodles, bread, and snacks. The animal protein groups included red meat, white meat, processed meat, other meat including meat by-products, fish and shellfish, processed and salted fish, eggs, and milk and dairy. The estimated energy requirement (EER) was calculated based on each participant's sex, age, weight, height, and physical activity level according to Dietary Reference Intakes for Koreans 2015 [31]. The physical activity level of all participants was considered to be low active according to a previous study [32].

2.3. Definition of Metabolic Syndrome

Metabolic syndrome was diagnosed if subjects had ≥3 of the following risk factors: (1) abdominal obesity (waist circumference ≥90 cm for men and ≥85 cm for women); (2) triglyceride (TG) levels ≥150 mg/dL; (3) high-density lipoprotein cholesterol (HDL-C) levels <40 mg/dL for men and <50 mg/dL for women; (4) systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg; and (5) fasting glucose levels ≥100 mg/dL. These standards are from the National Cholesterol Education Program Adult Treatment III [33], except for waist circumference, which was based on appropriate waist circumference threshold values for abdominal obesity in Koreans [34].

The anthropometric and clinical measurement data used for the diagnosis of metabolic syndrome were collected through the health examination by skilled clinical professional staffs with standardized protocols. The waist circumference was measured to the nearest 0.1 cm at the horizontal midpoint between the costal margin and the iliac crest. A blood sample was collected from each participant after an 8-h fast, immediately stored at 4 °C, and transported to cold storage at the Central Testing Institute in Seoul, Republic of Korea. Laboratory tests were conducted within one day after transport. The Hitachi Automatic Analyzer 7600-210 (Hitachi, Ltd., Tokyo, Japan) was used to measure TG, HDL-C, and plasma glucose levels. Blood pressure was measured three times in the right arm after the participant was seated quietly and resting for at least 5 min, using a mercury sphygmomanometer (Baumanometer®; Baum Co., Copiague, NY, USA). The average of the second and third measurements was used in this study.

2.4. Other Covariates

Socio-demographic data including age, sex, household income, and education level, and health-related lifestyle variables including drinking, smoking, and physical activity were collected through a health interview. Age groups were classified into 30–39, 40–49, and 50–64 years. Household income was categorized as the lowest, low middle, high middle, and highest. Education level was divided into ≤elementary school, middle school, high school, and ≥college/university. Among health behavior factors, drinking alcohol was divided into two groups based on the question of how often the participant had consumed alcohol over the past 1 year: ≤1/month and ≥2/month. Smoking was divided into two groups: participants were classified as a non-smoker if the participant had never smoked or had smoked in the past but did not currently smoke, and current smokers if they smoked occasionally or daily. Physical activities were evaluated with frequencies per week of high- and moderate-intensity physical activity, and walking >10 min. The categories were ≤1/week and ≥2/week depending on how frequently the participant did at least one activity of the above physical activities per week. All variables described above were used as covariates.

2.5. Statistical Analyses

Categorical variables of general characteristics were described as frequencies and proportions and compared by sex using the chi-square test. Continuous variables, such as energy and macronutrient

intake, and protein intake from major food sources of plant and animal protein were expressed as means and their standard errors and compared by sex using Student's *t*-test. Energy intake, and percent energy intake from carbohydrate, protein, and fat across quintiles of plant and animal protein intakes were presented as adjusted means and standard errors after adjusting for age, household income, education level, drinking, smoking, and physical activity. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated across quintiles of protein intake using the lowest quintile (Q1) as a reference by sex using multivariate logistic regression analysis to evaluate the association of plant and animal protein intakes with metabolic syndrome risk factors. In sub-analyses, the association of metabolic syndrome risk factors with animal protein intake according to levels of EER (≤EER and >EER) was assessed; ≤EER group includes participants who consume energy intake less than EER, and >EER group includes participants who consume energy intake more than EER. The *p*-value for trend across quintiles was assessed using the median protein intake (g/day) of each quintile. The factors such as age, energy intake, the percentage of energy intake from saturated fat, monounsaturated fat, and polyunsaturated fat, household income, education level, drinking, smoking, and physical activity were used as independent variables to adjust in the analysis.

All statistical analyses were performed using SAS software version 9.4 (SAS Institute, Cary, NC, USA) and the PROC SURVEY command in SAS, which incorporates a complex sampling design for a national survey. Survey sample weights, strata, and clusters were applied in the PROC SURVEY procedure.

3. Results

3.1. General Characteristics of Study Subjects

Table 1 shows the general characteristics of study subjects by sex. The proportion of women was higher than men (men: 39.4%; women: 60.6%; $p < 0.001$). Those aged 50–64 years were the highest percentage among all age groups in both men and women (men: 36.7%; women: 34.9%; $p < 0.001$). Men were more likely to have a higher household income ($p = 0.012$) and education level above college or university ($p < 0.001$) than women. Men tended to drink, smoke, and exercise more than women ($p < 0.001$). Men consumed more energy (kcal/day), carbohydrate, protein including both plant and animal protein, fat, and fatty acids ($p < 0.001$). The percent energy intake from animal protein (7.4% ± 0.1%) was higher than plant protein (6.9% ± 0.0%) in men, while that from animal protein (7.0% ± 0.1%) was lower than plant protein (7.4% ± 0.0%) in women ($p < 0.001$). However, the percent energy intake from protein was not significantly different between men and women ($p = 0.637$) and that from carbohydrate ($p < 0.001$) and fat ($p < 0.001$) including saturated fat ($p = 0.013$) and polyunsaturated fat ($p < 0.001$) was higher in women than men.

Table 1. General characteristics of study subjects by sex.

	Men	Women	*p*-Value [1]
***n* (%)**	5318 (39.4)	8167 (60.6)	<0.001
Demographic factors, *n* (%)			
Age			<0.001
30–39 years	1695 (31.9)	2476 (30.3)	
40–49 years	1673 (31.5)	2839 (34.8)	
50–64 years	1950 (36.7)	2852 (34.9)	
Household income			0.012
Lowest	358 (6.8)	653 (8.0)	
Low middle	1207 (22.8)	1940 (23.8)	
High middle	1789 (33.8)	2637 (32.4)	
Highest	1946 (36.7)	2916 (35.8)	
Education			<0.001
≤Elementary school	300 (6.2)	608 (7.9)	
Middle school	374 (7.7)	691 (9.0)	

Table 1. *Cont.*

	Men	Women	*p*-Value [1]
High school	1564 (32.1)	2881 (37.4)	
≥College/University	2630 (54.0)	3531 (45.8)	
Health behaviors, *n* (%)			
Drinking			<0.001
≤1/month	1597 (32.3)	4304 (59.5)	
≥2/month	3345 (67.7)	2934 (40.5)	
Smoking			<0.001
Non-smoker	2930 (57.3)	7558 (94.7)	
Current smoker	2180 (42.7)	425 (5.3)	
Physical activity			<0.001
≤1/week	3190 (65.6)	5603 (72.7)	
≥2/week	1673 (34.4)	2104 (27.3)	
Nutrient intake (mean ± SE)			
Energy (kcal/day)	2436.9 ± 13.6	1760.6 ± 8.4	<0.001
Carbohydrate (g/day)	347.6 ± 2.0	274.4 ± 1.4	<0.001
Protein (g/day)	87.6 ± 0.6	63.3 ± 0.4	<0.001
Plant protein (g/day)	40.9 ± 0.3	32.2 ± 0.2	<0.001
Animal protein (g/day)	46.6 ± 0.6	31.2 ± 0.3	<0.001
Fat (g/day)	56.2 ± 0.5	41.7 ± 0.3	<0.001
Saturated fat (g/day)	16.8 ± 0.2	12.4 ± 0.1	<0.001
Monounsaturated fat (g/day)	18.2 ± 0.2	13.3 ± 0.1	<0.001
Polyunsaturated fat (g/day)	13.8 ± 0.1	10.5 ± 0.1	<0.001
% Energy from (%E)			
Carbohydrate	58.8 ± 0.2	63.3 ± 0.2	<0.001
Protein	14.4 ± 0.1	14.4 ± 0.1	0.637
Plant protein	6.9 ± 0.0	7.4 ± 0.0	<0.001
Animal protein	7.4 ± 0.1	7.0 ± 0.1	<0.001
Fat	20.1 ± 0.1	20.7 ± 0.1	<0.001
Saturated fat	6.0 ± 0.0	6.2 ± 0.0	0.013
Monounsaturated fat	6.5 ± 0.1	6.6 ± 0.0	0.081
Polyunsaturated fat	5.0 ± 0.0	5.2 ± 0.0	<0.001

[1] *p*-values derived from chi-squared test for categorical variables and Student's *t*-test for continuous variables represent differences between men and women.

3.2. Major Food Sources of Plant and Animal Protein Intake

Major food sources of plant and animal protein intake of the study subjects are shown in Table 2. Among all food sources, the top protein source was refined grains for both men (19.5 ± 0.2 g/day) and women (14.3 ± 0.1 g/day), followed by red meat and fish and shellfish (men: 14.5 ± 0.3 and 9.1 ± 0.3 g/day; women: 8.7 ± 0.2 and 5.6 ± 0.2 g/day, respectively). Major food sources for plant and animal proteins were also the same between men and women. The major plant protein sources were refined grains, vegetables, and legume and legume products, and those of animal protein sources were red meat, fish and shellfish, and white meat, for both men and women. The absolute amount of protein intake from each food group, except for fruits ($p < 0.001$) and milk and dairy ($p < 0.001$), was higher in men than women.

Table 2. Major food sources of plant and animal protein intake in Korean adults aged 30–64 years by sex.

	Men	Women	*p*-Value [1]
Plant protein sources (g/day)			
Refined grains	19.5 ± 0.2	14.3 ± 0.1	<0.001
Other grains	2.6 ± 0.1	2.6 ± 0.1	0.887
Legume and legume products	3.8 ± 0.1	3.1 ± 0.1	<0.001
Vegetables	6.9 ± 0.1	5.5 ± 0.0	<0.001
Fruits	1.1 ± 0.0	1.4 ± 0.0	<0.001

Table 2. *Cont.*

	Men	Women	*p*-Value [1]
Condiments	3.2 ± 0.1	2.2 ± 0.0	<0.001
Nuts and Seeds	0.9 ± 0.0	0.8 ± 0.0	0.708
Animal protein sources (g/day)			
Red meat	14.5 ± 0.3	8.7 ± 0.2	<0.001
White meat	6.7 ± 0.3	4.1 ± 0.2	<0.001
Processed meat	3.3 ± 0.2	2.0 ± 0.1	<0.001
Other meat	1.1 ± 0.1	0.5 ± 0.0	<0.001
Fish and shellfish	9.1 ± 0.3	5.6 ± 0.2	<0.001
Processed and salted fish	4.6 ± 0.1	3.4 ± 0.1	<0.001
Eggs	4.2 ± 0.1	3.5 ± 0.1	<0.001
Milk and dairy	2.5 ± 0.1	2.9 ± 0.1	<0.001

All values are presented as means ± standard errors and derived from a regression model. [1] *p*-values derived from Student's *t*-test represent differences between men and women.

Overall, the proportion of protein intake from each food group among total protein intake (protein intake from each food group/total protein intake × 100) was different between men and women by types of food sources (Figure 1). The proportion of protein intake from plant food sources was higher in women than men except for condiments ($p < 0.05$). The proportion of protein intake from refined grains was not significantly different between men and women (men: 25.3%; women: 25.1%). Among animal food sources, the proportions of protein intake from red meat ($p < 0.001$), white meat ($p < 0.001$), processed meat ($p < 0.05$), other meat ($p < 0.001$), and fish and shellfish ($p < 0.001$) were higher in men than women, while those from eggs (men: 4.9%; women: 5.6%; $p < 0.001$), and milk and dairy (men: 3.0%; women: 4.8%; $p < 0.001$) were higher in women than men. In summary, most plant food sources and several animal food sources, particularly eggs, and milk and dairy, accounted for a higher proportion of total protein intake in women than men, while meat and fish accounted for a greater proportion in men than women.

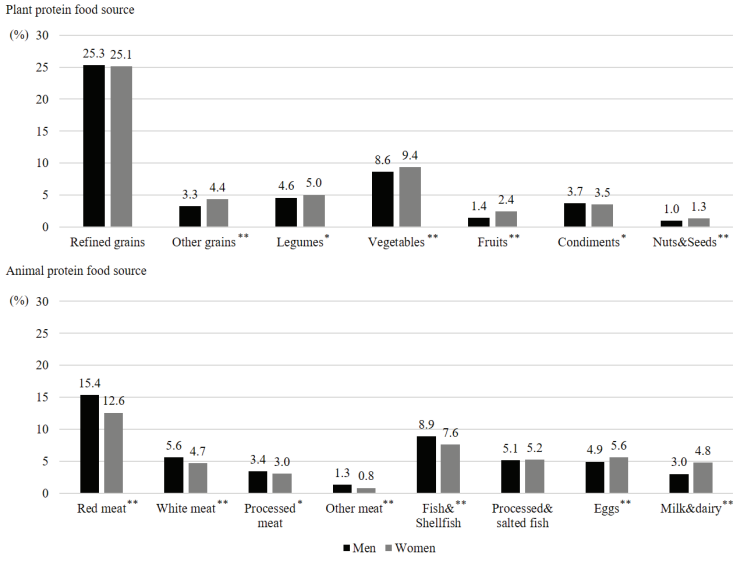

Figure 1. The proportion of protein intake from plant and animal food sources among total protein intake by each food group in Korean adults aged 30–64 years. *p*-values derived from Student's *t*-test represent differences between men and women (* $p < 0.05$, ** $p < 0.001$).

3.3. Energy and Nutrient Intakes According to Plant and Animal Protein Intake Levels

Energy and percent energy intakes from major nutrients according to the quintiles of intake of types of protein food sources are presented in Table 3. Energy intake increased across all types of protein intake levels in both men and women (p for trend < 0.001). The percent energy intake from protein and fat increased across animal protein intake quintile groups in both men and women (p for trend < 0.001); however, they remained almost constant regardless of incremental plant protein intake in both men and women. The percent energy intake from protein (Q5: 18.5% ± 0.2% for men, 18.7% ± 0.2% for women) and fat (Q5: 23.1% ± 0.4% for men, 24.7% ± 0.3% for women) was higher in the highest quintile group of animal protein intake than that of plant protein intake (protein Q5: 13.7% ± 0.2% for men, 14.2% ± 0.2% for women; fat Q5: 18.2% ± 0.3% for men, 19.5% ± 0.3% for women) in both men and women. Hence, the percent energy intake from carbohydrate intake decreased across animal protein intake quintile groups (p for trend < 0.001), while that increased across plant protein intake quintile groups in both men and women (p for trend < 0.001).

3.4. Association of Plant and Animal Protein Intake with Metabolic Syndrome

Table 4 shows the association of protein intake by types of food sources with metabolic syndrome in Korean men and women. Positive associations of animal protein intake with abdominal obesity, reduced HDL-C, and elevated fasting glucose were observed in men. Those in the highest quintile group of animal protein intake had a higher prevalence of abdominal obesity (OR: 1.30, 95% CI: 1.00–1.70), reduced HDL-C (OR: 1.43, 95% CI: 1.07–1.90), and elevated fasting glucose (OR: 1.32, 95% CI: 1.01–1.74) than the lowest quintile group. However, the positive trend was significant only in reduced HDL-C (p for trend = 0.009). Men in the highest quintile group of plant protein intake had a lower prevalence of increased blood pressure compared to the lowest quintile group (OR: 0.71, 95% CI: 0.54–0.94, p for trend = 0.047). Neither animal nor plant protein intakes were significantly associated with any of the metabolic syndrome risk factors in women.

To confirm whether the energy intake level affects the association of animal protein intake with metabolic syndrome, the subjects were further divided by EER (Table 5). Men in the highest quintile group of animal protein intake and who had energy intake ≤ EER had an increased prevalence of abdominal obesity (OR: 1.60, 95% CI: 1.11–2.31, p for trend = 0.003) compared to the lowest quintile group. In addition, the association of reduced HDL-C with animal protein intake also showed a positive linear trend in ≤ EER group (p for trend = 0.047) in men. Animal protein intake was not significantly associated with metabolic syndrome risk factors regardless of the energy intake level in women.

Table 3. Energy and nutrient intakes according to quintiles of plant and animal protein intake in Korean adults aged 30–64 years.

	Q1	Q2	Q3	Q4	Q5	*p* for Trend [1]
Men						
Plant protein (*n* = 5318)	1063	1064	1064	1064	1063	
Energy (kcal/day)	1636.1 ± 28.0	2031.4 ± 27.8	2333.4 ± 27.4	2652.7 ± 29.1	3136.9 ± 30.6	<0.001
Energy from carbohydrate (%E)	57.3 ± 0.7	60.9 ± 0.6	61.9 ± 0.6	62.3 ± 0.5	63.7 ± 0.5	<0.001
Energy from protein (%E)	13.8 ± 0.2	13.7 ± 0.2	13.8 ± 0.2	13.9 ± 0.2	13.7 ± 0.2	0.901
Energy from fat (%E)	18.5 ± 0.4	18.7 ± 0.3	18.6 ± 0.3	18.6 ± 0.3	18.2 ± 0.3	0.381
Animal protein (*n* = 5277)	1055	1056	1055	1056	1055	
Energy (kcal/day)	1807.3 ± 30.1	2082.6 ± 27.3	2321.1 ± 30.0	2586.5 ± 29.6	3129.7 ± 33.2	<0.001
Energy from carbohydrate (%E)	69.4 ± 0.5	64.6 ± 0.5	60.6 ± 0.5	55.8 ± 0.5	48.8 ± 0.5	<0.001
Energy from protein (%E)	11.0 ± 0.1	12.7 ± 0.1	13.9 ± 0.1	15.2 ± 0.1	18.5 ± 0.2	<0.001
Energy from fat (%E)	15.5 ± 0.3	17.0 ± 0.3	19.2 ± 0.3	20.8 ± 0.3	23.1 ± 0.4	<0.001
Women						
Plant protein (*n* = 8167)	1633	1634	1633	1634	1633	
Energy (kcal/day)	1152.2 ± 18.2	1470.6 ± 19.0	1736.0 ± 18.6	1982.1 ± 20.7	2510.3 ± 24.2	<0.001
Energy from carbohydrate (%E)	59.9 ± 0.6	62.4 ± 0.5	63.3 ± 0.5	64.1 ± 0.5	63.5 ± 0.5	<0.001
Energy from protein (%E)	14.4 ± 0.2	14.2 ± 0.2	13.9 ± 0.2	13.8 ± 0.2	14.2 ± 0.2	0.148
Energy from fat (%E)	20.6 ± 0.4	19.9 ± 0.3	19.5 ± 0.4	19.3 ± 0.3	19.5 ± 0.3	0.001
Animal protein (*n* = 8105)	1621	1621	1621	1621	1621	
Energy (kcal/day)	1363.5 ± 22.5	1519.5 ± 24.1	1650.6 ± 23.1	1861.9 ± 24.1	2293.8 ± 27.3	<0.001
Energy from carbohydrate (%E)	71.3 ± 0.4	66.1 ± 0.4	62.6 ± 0.4	58.5 ± 0.4	51.6 ± 0.5	<0.001
Energy from protein (%E)	11.0 ± 0.1	12.6 ± 0.1	13.9 ± 0.1	15.5 ± 0.1	18.7 ± 0.2	<0.001
Energy from fat (%E)	15.2 ± 0.3	18.1 ± 0.3	19.9 ± 0.3	21.9 ± 0.3	24.7 ± 0.3	<0.001

All values are presented as means ± standard errors and derived from a multivariate regression model after adjustment for age, household income, education level, drinking, smoking, and physical activity. [1] *p* for trend was derived from a multivariate regression model using the median protein intake (g/day) for each quintile as the continuous variable.

Table 4. Odds ratios and 95% confidence intervals for metabolic syndrome parameters [1] according to quintiles of plant and animal protein intake in Korean adults aged 30–64 years.

	Q1	Q2	Q3	Q4	Q5	*p* for Trend [2]
Men						
Plant protein intake (*n* = 5318)	1063	1064	1064	1064	1063	
Median intake (g/day)	22.2 ± 0.2	31.2 ± 0.1	39.0 ± 0.1	47.8 ± 0.2	62.5 ± 0.4	
Median intake (%E)	5.3 ± 0.1	6.3 ± 0.1	6.7 ± 0.1	7.3 ± 0.1	8.3 ± 0.1	
Abdominal obesity	Ref	0.88 (0.70–1.09)	0.85 (0.68–1.07)	1.04 (0.82–1.32)	1.07 (0.81–1.41)	0.313
Elevated TG	Ref	0.98 (0.80–1.20)	0.90 (0.72–1.13)	0.81 (0.63–1.04)	0.94 (0.71–1.23)	0.423
Reduced HDL-C	Ref	0.80 (0.63–1.02)	0.75 (0.58–0.96)	0.78 (0.59–1.03)	0.90 (0.66–1.24)	0.678
Increased blood pressure	Ref	0.79 (0.64–0.98)	0.75 (0.60–0.93)	0.79 (0.63–1.00)	0.71 (0.54–0.94)	0.047
Elevated fasting glucose	Ref	0.91 (0.73–1.14)	0.99 (0.79–1.24)	0.83 (0.64–1.07)	0.98 (0.75–1.30)	0.818
Metabolic syndrome	Ref	0.88 (0.70–1.11)	0.77 (0.61–0.99)	0.84 (0.65–1.10)	0.90 (0.67–1.20)	0.574
Animal protein intake (*n* = 5277)	1055	1056	1055	1056	1055	
Median intake (g/day)	11.4 ± 0.3	25.3 ± 0.2	37.0 ± 0.3	53.9 ± 0.3	88.4 ± 1.3	
Median intake (%E)	2.5 ± 0.1	4.9 ± 0.1	6.5 ± 0.1	8.6 ± 0.1	12.1 ± 0.2	
Abdominal obesity	Ref	1.32 (1.05–1.68)	1.15 (0.90–1.46)	1.41 (1.11–1.81)	1.30 (1.00–1.70)	0.101
Elevated TG	Ref	1.07 (0.87–1.32)	0.91 (0.73–1.13)	1.05 (0.84–1.31)	0.91 (0.71–1.17)	0.414
Reduced HDL-C	Ref	1.24 (0.98–1.56)	1.07 (0.83–1.38)	1.54 (1.19–2.00)	1.43 (1.07–1.90)	0.009
Increased blood pressure	Ref	1.02 (0.81–1.27)	0.99 (0.79–1.23)	0.97 (0.77–1.23)	0.92 (0.71–1.19)	0.576
Elevated fasting glucose	Ref	1.33 (1.07–1.64)	1.28 (1.02–1.60)	1.29 (1.02–1.64)	1.32 (1.01–1.74)	0.138
Metabolic syndrome	Ref	1.51 (1.19–1.91)	1.27 (0.99–1.64)	1.45 (1.12–1.90)	1.28 (0.96–1.71)	0.391
Women						
Plant protein intake (*n* = 8167)	1633	1634	1633	1634	1633	
Median intake (g/day)	16.4 ± 0.1	23.9 ± 0.1	30.0 ± 0.1	37.4 ± 0.1	50.5 ± 0.3	
Median intake (%E)	5.8 ± 0.1	6.8 ± 0.0	7.2 ± 0.1	7.9 ± 0.1	8.8 ± 0.1	
Abdominal obesity	Ref	0.82 (0.65–1.04)	0.85 (0.66–1.09)	1.03 (0.79–1.34)	0.89 (0.64–1.23)	0.964
Elevated TG	Ref	0.95 (0.75–1.21)	0.85 (0.66–1.10)	0.89 (0.68–1.17)	0.89 (0.64–1.23)	0.496
Reduced HDL-C	Ref	0.98 (0.80–1.19)	1.21 (0.98–1.49)	1.12 (0.91–1.38)	1.01 (0.78–1.32)	0.774
Increased blood pressure	Ref	1.03 (0.81–1.32)	0.82 (0.63–1.07)	0.77 (0.57–1.03)	0.87 (0.62–1.22)	0.229
Elevated fasting glucose	Ref	1.09 (0.88–1.35)	1.15 (0.91–1.46)	0.98 (0.77–1.26)	1.04 (0.77–1.41)	0.911
Metabolic syndrome	Ref	0.87 (0.66–1.15)	0.94 (0.70–1.27)	0.87 (0.64–1.18)	0.92 (0.62–1.35)	0.723
Animal protein intake (*n* = 8105)	1621	1621	1621	1621	1621	
Median intake (g/day)	7.3 ± 0.2	16.5 ± 0.1	25.3 ± 0.1	36.5 ± 0.2	61.0 ± 0.5	
Median intake (%E)	2.1 ± 0.1	4.5 ± 0.1	6.2 ± 0.1	8.1 ± 0.1	11.6 ± 0.2	
Abdominal obesity	Ref	0.77 (0.61–0.98)	0.84 (0.66–1.08)	0.83 (0.65–1.08)	0.80 (0.60–1.07)	0.301

Table 4. *Cont.*

	Q1	Q2	Q3	Q4	Q5	*p* for Trend [2]
Elevated TG	Ref	0.92 (0.73–1.15)	0.84 (0.66–1.07)	0.99 (0.77–1.27)	0.85 (0.64–1.13)	0.484
Reduced HDL-C	Ref	0.85 (0.70–1.03)	0.83 (0.68–1.00)	0.91 (0.74–1.11)	0.87 (0.70–1.09)	0.456
Increased blood pressure	Ref	0.80 (0.63–1.01)	0.86 (0.67–1.11)	0.79 (0.61–1.03)	0.83 (0.62–1.11)	0.502
Elevated fasting glucose	Ref	0.97 (0.78–1.21)	1.06 (0.84–1.33)	1.01 (0.79–1.29)	1.00 (0.76–1.31)	0.926
Metabolic syndrome	Ref	0.88 (0.67–1.15)	0.84 (0.63–1.10)	0.98 (0.74–1.30)	0.81 (0.58–1.14)	0.386

All values (ORs and 95% CIs) were derived from a multivariate logistic regression model after adjustment for age, energy intake, the percentage of energy intake from saturated fat, monounsaturated fat, and polyunsaturated fat, household income, education level, drinking, smoking, and physical activity. [1] Metabolic syndrome definition: (1) abdominal obesity (waist circumference ≥ 90 cm for men and ≥85 cm for women); (2) triglyceride (TG) levels ≥ 150 mg/dL; (3) high-density lipoprotein cholesterol (HDL-C) levels < 40 mg/dL for men and <50 mg/dL for women; (4) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; (5) fasting glucose levels ≥ 100 mg/dL. [2] *p* for trend was derived from a multivariate logistic regression model using the median protein intake (g/day) for each quintile as the continuous variable.

Table 5. Odds ratios and 95% confidence intervals for metabolic syndrome parameters [1] according to quintiles of animal protein intake and the level of estimated energy requirements (EER) [2] in Korean men aged 30–64 years.

Animal Protein Intake	Q1	Q2	Q3	Q4	Q5	*p* for Trend [3]
Men (*n* = 5277)	1055	1056	1055	1056	1055	
≤EER (*n* = 3106)	862	789	658	531	266	
Abdominal obesity	Ref	1.26 (0.97–1.64)	1.15 (0.56–1.53)	1.54 (1.15–2.07)	1.60 (1.11–2.31)	0.003
Elevated TG	Ref	1.09 (0.85–1.40)	0.91 (0.69–1.20)	1.00 (0.75–1.33)	0.83 (0.57–1.19)	0.333
Reduced HDL-C	Ref	1.23 (0.95–1.60)	1.17 (0.87–1.58)	1.67 (1.22–2.29)	1.26 (0.81–1.94)	0.047
Increased blood pressure	Ref	0.97 (0.75–1.27)	0.92 (0.70–1.20)	0.94 (0.69–1.28)	0.95 (0.64–1.42)	0.929
Elevated fasting glucose	Ref	1.18 (0.92–1.52)	1.22 (0.92–1.62)	1.32 (0.97–1.78)	1.28 (0.89–1.86)	0.087
Metabolic syndrome	Ref	1.43 (1.08–1.89)	1.31 (0.97–1.76)	1.66 (1.21–2.29)	1.25 (0.84–1.87)	0.082
>EER (*n* = 2171)	193	267	397	525	789	
Abdominal obesity	Ref	1.09 (0.60–1.99)	0.76 (0.43–1.34)	0.89 (0.51–1.54)	0.87 (0.51–1.48)	0.714
Elevated TG	Ref	0.96 (0.61–1.50)	0.91 (0.58–1.41)	1.12 (0.72–1.74)	0.97 (0.63–1.51)	0.920
Reduced HDL-C	Ref	1.36 (0.76–2.42)	0.96 (0.53–1.71)	1.54 (0.89–2.69)	1.55 (0.89–2.72)	0.057
Increased blood pressure	Ref	1.22 (0.73–2.04)	1.26 (0.77–2.06)	1.14 (0.71–1.84)	1.05 (0.66–1.67)	0.526
Elevated fasting glucose	Ref	1.94 (1.22–3.10)	1.48 (0.94–2.33)	1.43 (0.92–2.23)	1.60 (1.00–2.54)	0.423
Metabolic syndrome	Ref	1.71 (0.98–3.00)	1.19 (0.70–2.05)	1.20 (0.70–2.07)	1.24 (0.71–2.15)	0.868

All values (ORs and 95% CIs) were derived from a multivariate logistic regression model after adjustment for age, energy intake, the percentage of energy intake from saturated fat, monounsaturated fat, and polyunsaturated fat, household income, education level, drinking, smoking, and physical activity. [1] Metabolic syndrome definition: (1) abdominal obesity (waist circumference ≥ 90 cm for men and ≥85 cm for women); (2) triglyceride (TG) levels ≥ 150 mg/dL; (3) high-density lipoprotein cholesterol (HDL-C) levels < 40 mg/dL for men and <50 mg/dL for women; (4) systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; (5) fasting glucose levels ≥ 100 mg/dL. [2] ≤EER: energy intake less than EER; >EER: energy intake more than EER. [3] *p* for trend was derived from a multivariate logistic regression model using the median protein intake (g/day) for each quintile as the continuous variable.

4. Discussion

This study found that animal protein intake was positively associated with abdominal obesity, reduced HDL-C, and elevated fasting glucose regardless of fatty acid intake level in men, not in women, based on the results of data analysis of 13,485 middle-aged Korean adults who participated in the 2013–2018 KNHANES. Furthermore, stronger associations of animal protein intake with abdominal obesity were shown in men who consumed less than EER. Plant protein intake was negatively associated with increased blood pressure in men. Neither animal nor plant protein intakes were significantly associated with any of the metabolic syndrome risk factors in women.

The adverse effects of animal protein intake on abdominal obesity and cholesterol levels in men have been observed in previous studies. Animal protein intake or intakes from meat and fish were positively associated with the risk of abdominal obesity and general obesity [35–37] among male adults according to studies conducted in Western countries. In a Chinese longitudinal study, red meat consumption was also positively associated with an increased waist circumference in men only [38]. In a European study, high meat consumption was associated with increased cholesterol levels [39]. However, plant protein intake was inversely associated with waist circumference and weight change in the Western population [35], contrary to this study showing that plant protein intake was not associated with abdominal obesity. In summary, higher animal protein intake including meat consumption also seems to be generally associated with an increased risk of abdominal obesity and cholesterol levels in other populations; however, the effect of plant protein intake on those parameters in various populations is still inconclusive.

On the other hand, favorable effects of animal protein intake on other metabolic syndrome risk factors have been observed in several Asian populations. According to a study with rural Chinese population, increased animal protein intake was related to a significant decrease in blood pressure in women, whereas no significant association was observed for plant protein intake [40]. It has been also observed that incremental animal protein intake was more strongly related to a reduction in blood pressure than plant protein intake in Japanese adults [41]. In contrast, several Western studies have shown that animal protein intake had no significant effect on blood pressure [42,43].

One of the possible explanations for the inconsistent effects of animal protein intake on metabolic parameters in different populations might be that different proportions of animal and plant protein intake result in different diet quality. Korean men had more similar proportions of plant (51.5%) and animal (48.4%) protein intake, while women had a higher proportion of plant (54.9%) than animal (45.1%) protein intake according to this study. The high proportion of animal protein intake of Korean men found in this study is similar to the Western dietary pattern, where the percentage of protein intake from meat and dairy foods is higher (62%) than plant foods (30%) in American adults [44]. The percent energy intake from animal protein intake was also higher than that from plant protein intake in men (animal: 7.4%; plant: 6.9%), consistent with Western adult males (animal: 10.9%; plant: 6.5%) [35]. Thus, it is suggested that high animal protein intake of men in this study may result in a similar adverse effect on metabolic parameters. On contrary, the proportion of animal protein intake was very low (20%) in a Chinese rural population, and was negatively associated with a metabolic syndrome risk factor [40]. Therefore, the association between protein intakes derived from different food sources and metabolic diseases requires more attention.

Underlying mechanisms for the effect of protein intake on metabolic disorders can be explained by amino acid metabolism. For example, methionine, which is mainly from animal foods, has been shown in several in vivo studies to have atherogenic effects by inducing vitamin B deficiency, hyperhomocysteinemia, and atherosclerosis and thus is linked to an increased risk of acute coronary syndrome [45]. The other amino acids, such as glycine, tryptophan, and tyrosine, which are also abundant in animal protein, have been reported to improve blood pressure by modulating vascular tone or endothelial function, or by changing specific neuronal pathways [46,47]. Glutamic acid, which is predominantly in plants, was associated with lower arterial stiffness [48]. Thus, a different amino acid

composition from plant and animal sources could play an important role in the association between animal protein and metabolic diseases and further related study are required to confirm.

Another issue is that the effects of protein intake on metabolic syndrome risk factors between younger adults and the elderly in Korea are dissimilar. Contrary to our findings, several metabolic syndrome risk factors such as abdominal obesity, hypertriglyceridemia, and high fasting blood glucose were inversely associated with protein intake among the Korean elderly [24–26]. According to previous studies, the elderly aged 60 years or older consumed only 30% protein from animal foods [24,29], while adults aged 30–64 years consumed about 47% protein from animal foods as observed in this study. In addition, the Korean elderly aged 60 years or older consumed about 20 g less protein per day [24,29] than adults aged 30–64 years in this study. Thus, age and energy intake as well as potential confounding variables such as physical activity, drinking, and smoking, etc. were adjusted in all analyses. A comparative analysis of the association of protein intake by food sources with metabolic diseases between younger adults and the elderly should be considered in further studies.

This study had several limitations. First, it was not able to establish a causal relationship of protein intake with metabolic syndrome due to the cross-sectional design. Second, protein intake assessed in this study could not have fully reflected the usual protein intake since we used only one 24-h dietary recall dataset. Third, this study was not able to assess the effect of variations of other macronutrient intakes such as fat and carbohydrate intake on the association of protein intake with metabolic syndrome even though the other macronutrient intake generally changes as protein intake increases. According to our findings, fat intake was positively associated with animal protein intake while carbohydrate intake was negatively associated with animal protein intake. Adjustment with fatty acid intake was conducted to attenuate the effect of fat intake on the association of animal protein intake with metabolic syndrome, however, an in-depth analysis that assesses variations in both fat and carbohydrate intakes with increments of protein intake is still required in future studies. In addition, amino acids from animal food that can influence the risk of metabolic diseases were not analyzed in the study due to lack of database, thus the interactions between animal protein or amino acid intakes on the metabolic diseases also need to be clarified in future studies. Despite these limitations, this study is the first study to analyze the association of protein intake from food sources with metabolic syndrome risk factors using a large sample of middle-aged Korean adults.

5. Conclusions

In summary, the animal protein intake of middle-aged Korean men was positively associated with abdominal obesity, reduced HDL-C, and elevated fasting glucose regardless of fatty acid intake level and stronger associations of animal protein intake with abdominal obesity were shown in men who consumed less than EER, while plant protein intake was negatively associated with increased blood pressure. Neither animal nor plant protein intakes were significantly associated with any of the metabolic syndrome risk factors in women. Our findings imply that lower animal protein intake may be a beneficial factor for metabolic syndrome management, particularly for abdominal obesity, reduced HDL-C, and elevated fasting glucose in middle-aged Korean men.

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Association of Dietary Patterns and Type-2 Diabetes Mellitus in Metabolically Homogeneous Subgroups in the KORA FF4 Study

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Abstract: There is evidence that a change in lifestyle, especially physical activity and diet, can reduce the risk of developing type-2 diabetes mellitus (T2DM). However, the response to dietary changes varies among individuals due to differences in metabolic characteristics. Therefore, we investigated the association between dietary patterns and T2DM while taking into account these differences. For 1287 participants of the population-based KORA FF4 study (Cooperative Health Research in the Region of Augsburg), we identified three metabolically-homogenous subgroups (metabotypes) using 16 clinical markers. Based on usual dietary intake data, two diet quality scores, the Mediterranean Diet Score (MDS) and the Alternate Healthy Eating Index (AHEI), were calculated. We explored the associations between T2DM and diet quality scores. Multi-variable adjusted models, including metabotype subgroup, were fitted. In addition, analyses stratified by metabotype were carried out. We found significant interaction effects between metabotype and both diet quality scores ($p < 0.05$). In the analysis stratified by metabotype, significant negative associations between T2DM and both diet quality scores were detected only in the metabolically-unfavorable homogenous subgroup (Odds Ratio (OR) = 0.62, 95% confidence interval (CI) = 0.39–0.90 for AHEI and OR = 0.60, 95% CI = 0.40–0.96 for MDS). Prospective studies taking metabotype into account are needed to confirm our results, which allow for the tailoring of dietary recommendations in the prevention of T2DM.

Keywords: diabetes mellitus; dietary pattern; metabotype; metabolic phenotype; Mediterranean Diet Score; Alternate Healthy Eating Index

1. Introduction

During the past decades, the prevalence of type-2 diabetes mellitus (T2DM) has rapidly and dramatically increased [1]. This poses a major public health problem, especially in Western societies. It is estimated that the number of diabetic adults worldwide will increase by 50% to a total of 693 million by 2045 [2]. According to the International Diabetes Federation, about 50% of T2DM cases are undetected [2]. These numbers clearly highlight the ever-increasing severity of the diabetes epidemic and the pressing need to investigate its underlying pathophysiology so that better therapeutic strategies can be developed.

Prediabetes and diabetes mellitus are associated with an increased risk of complications, namely cardiovascular disease (CVD), retinopathy, neuropathy, and nephropathy [3]. Besides a lack of sufficient physical activity, a major role in the development of diabetes mellitus is attributed to diet [4,5]. A change in lifestyle can reduce the relative risk of developing diabetes mellitus by 40–70% in prediabetic persons [6]. Thus, the thorough investigation of dietary habits and their effects on the development of chronic diseases can have a major impact on public health.

In traditional approaches, single food groups or nutrients are examined with respect to their associations with certain diseases. Yet in reality, food groups and nutrients are not consumed in isolation. Therefore, nutritional epidemiology nowadays commonly examines dietary patterns. Dietary patterns make it possible to capture a comprehensive picture of a person's overall diet and thereby consider possible interactions between nutrients or food groups [7,8]. A diet quality score is a dietary pattern defined a priori and is a useful tool to measure the overall quality of a diet. Two frequently used diet quality scores are the Alternate Healthy Eating Index (AHEI) [9] and the Mediterranean Diet Score (MDS) [10]. Dietary patterns have been consistently associated with T2DM in various studies investigating different ethnic populations, sexes or age groups [11–22], although metabolic characteristics have so far not been comprehensively accounted for.

Interindividual differences in metabolism and dietary requirements are driven by intrinsic as well as extrinsic factors, e.g., (epi-)genetic background, body composition, and lifestyle [23–27]. The identification of metabotypes, i.e., metabolically-homogenous subgroups, has been established to adequately incorporate this knowledge when modelling diet-disease associations and in the context of personalized nutrition [28–35]. The metabotype identification consists of comparing individuals' metabolic characteristics to each other and grouping similar individuals together. They are assigned the same metabotype. Selecting which parameters should be included in the process of defining metabotypes offers the possibility to encompass a variety of important biological processes. However, one major limitation of the concept is that there is still no unique definition of the term "metabotype" available, nor a single way to identify homogenous groups. Our group is currently investigating the selection of an optimal set of biochemical markers. A previously-published study by our group showed differential associations between single foods groups and T2DM across different metabotypes, underlining the importance of taking metabotype information into account [33].

The aim of the present study is therefore to examine the associations between dietary patterns and T2DM while accounting for metabotype. This is of special interest when developing targeted nutritional recommendations.

2. Materials and Methods

2.1. Study Population

The Cooperative Health Research in the Region of Augsburg (KORA) FF4 study is a cross-sectional, population-based study that was conducted in the Bavarian region of Augsburg in 2013/2014. It is the second follow-up of the KORA S4 survey that took place from 1999 to 2001. Details on S4 as well as FF4 have been published previously [36,37]. Briefly, 2279 individuals, the vast majority with Caucasian ethnicity, participated in the KORA FF4 study. They were physically examined,

answered self-administered questionnaires, and participated in computer-assisted face-to-face interviews during their study center visit. Fasting blood samples were taken at the study center and immediately underwent preprocessing. For our analyses, we excluded participants without dietary information (n = 677, leaving 1602), without metabotype information (n = 41, leaving 1561), with type 1 diabetes (n = 3, leaving 1558), unclear glucose tolerance status due to missing oral glucose tolerance test (OGTT) information (n = 38, leaving 1520), or unclear/unvalidated glucose tolerance status (n = 1, leaving 1519). Furthermore, participants with missing information on their hypertensive status (n = 2, leaving 1517), and those with severe diseases, namely myocardial infarction (n = 46) and/or stroke (n = 34) and/or cancer (n = 158) were also excluded. The final data set for the analysis included 1287 participants.

The KORA studies were approved by the Ethics Committee of the Bavarian Chamber of Physicians and carried out in accordance with the Declaration of Helsinki (EK Nr. 06068, 25.10.2012). Written informed consent was obtained from all participants.

2.2. Covariates

The analyses described below included age (in years) and waist circumference (in cm) as continuous variables, sex (male/female), smoking status (never/former/current), education (< 10 years/10–12 years/> 12 years), physical activity (active/inactive) and actual hypertensive status (yes/no) as categorical covariates. The categorization of the education variable represents the German education system. Physically-active participants are those who were active during summer and winter and at the same time active for at least 1 h per week during at least one season. Hypertensive individuals included those with measured systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg and/or use of anti-hypertensive medication given that the subjects were aware that they had hypertension.

2.3. Metabotypes

Participants were grouped into three metabolically-homogenous subgroups. Details are given in References [34] and [33]. Briefly, a set of 16 clinical markers (body mass index (BMI), 12 laboratory parameters measured in serum, i.e., glucose, total cholesterol, high-density lipoprotein cholesterol, total cholesterol/high-density lipoprotein cholesterol ratio, low-density lipoprotein cholesterol, uric acid, triglycerides, gamma-glutamyltransferase, glutamate-pyruvate transaminase, glutamate-oxaloacetate transaminase, alkaline phosphatase high-sensitivity C-reactive protein and insulin, and two parameters measured in fresh venous whole EDTA blood, i.e., leukocytes and glycated hemoglobin) was used to identify and characterize three clusters by applying the k-means clustering algorithm. These 16 clinical markers are the available subset of markers originally used in the development of this metabotype concept. The prevalence and incidence of metabolic diseases in the clusters are used to quantify how well the clinical markers define distinct clusters. Missing observations were imputed by MICE (multivariate imputation by chained equations) and values were standardized prior to running the clustering algorithm. Metabotypes were derived in a larger sample of 2218 (out of 2279 total) FF4 participants. Only participants who did not fast for at least 8 h before blood collection (n = 54) or participants with more than 10% missing values of all clustering variables (n = 7) were excluded. The three cluster solution presented in Reference [33] describes cluster 3 as the most unfavorable metabotype with respect to the metabolic characteristics, e.g., the highest median concentrations of serum glucose and glycated hemoglobin, as well as other risk factors for T2DM, including age, obesity, physical inactivity, family history of diabetes and hypertension. In contrast, clusters 1 and 2 define rather beneficial metabotypes; cluster 1 shows even healthier characteristics than cluster 2 which can be considered as an "intermediate" cluster.

2.4. Dietary Intake and Diet Quality Scores

Usual dietary intake was calculated based on one food frequency questionnaire (FFQ) and up to three 24 h food lists (24HFL) per participant. One 24HFL was filled in by the participants during

their study center visit, and up to two further 24HFLs were filled in at home within the following three months. In contrast to that assessment of the short-term diet, the FFQ assesses the participants' diet over the last 12 months. Details are given elsewhere [38]. Briefly, a two-step approach was used to model consumption probabilities and consumption amounts. Usual intake estimates for the food items were further categorized into food groups based on the EPIC-Soft classification system [39]. Furthermore, estimates of nutrient intake were derived for every participant by linking the usual food group intake to the National Nutrient Database (Bundeslebensmittelschlüssel 3.02).

As measures of quality of the overall diet of the participants, the Alternate Healthy Eating Index (AHEI) 2010 [9] and the Mediterranean Diet Score (MDS) 2003 [10] were calculated based on the usual intake estimates. The AHEI 2010 consists of 11 food components, namely vegetables, fruits, whole grains, sugar-sweetened beverages (SSB) and fruit juice, nuts and legumes, red/processed meat, trans fat, long-chain (n-3) fatty acids, polyunsaturated fatty acids (PUFA), sodium and alcohol. Dietary intake of fish and shellfish was used as a proxy for long-chain (n-3) fatty acid intake. Based on the quantity of each food component consumed, 0 to 10 points were attributed, yielding a theoretical range of 0–110 points for the total AHEI score. The higher the score, the healthier the diet is considered to be. Cut-off values which result in minimum and maximum points per dietary component are given, and intermediate intakes are scored proportionally between those limits. For example, the maximum score of 10 is attributed if at least 5 servings of vegetables per day were consumed. Scoring servings proportionally would, therefore, attribute 2 points for every serving, that is 4 points for 2 servings, 6 points for 3, and so on. These cut-offs have been updated in the 2010 version of the original AHEI [40] according to state-of-the-art practices in nutritional science. In our analysis, the usual intake in g/day had to be converted to servings per day by using standard portion sizes as reported in Reference [9]. For example, one serving of green leafy vegetables equals 1 cup or 236.59 g and for all other vegetables, 1 serving equals 0.5 cup or 118.295 g. Furthermore, we had to exclude the trans-fat component, as this information is not available in our data set. For this reason, the modified AHEI computed in the present study with 10 components ranged theoretically from 0 to 100 points. Table 1 summarizes the components of the modified AHEI and the cut-off values applied in the analysis.

The MDS 2003 includes 9 dietary components, namely vegetables, legumes, fruits and nuts, cereals, fish, meat, dairy products, alcohol, and fat intake (ratio of monounsaturated to saturated fatty acids), where fish has been additionally included to the index originally developed [41]. For each of the components listed above, a value of either 0 or 1 point was assigned to each participant. Except for alcohol intake, sex-specific medians were used as cut-off values. Apart from the components of meat and dairy products, participants with a usual intake above the median for a given component were assigned a score of 1 and those below a score of 0. For the components of meat and dairy products, participants above the median were assigned to a score of 0 and below to a score of 1. Alcohol intakes between 10 and 50 g per day for men and between 5 and 25 g per day for women were considered as optimal, therefore leading to a score of 1 for this component. The total MDS calculated in the present study ranged from 0 to 9 points, where 0 reflects a minimal adherence and 9 reflects a strong adherence to the traditional Mediterranean diet. Table 2 summarizes the sex-specific cut-offs applied in our study.

Table 1. Criteria for the definition of the modified Alternate Healthy Eating Index [42].

Component	Criteria for Minimum Score (0)	Criteria for Maximum Score (10)
Vegetables, servings/d	0	≥5
Fruits, servings/d	0	≥4
Whole grains, g/d		
Women	0	75
Men	0	90
SSB and fruit juice, servings/d	≥1	0
Nuts and legumes, servings/d	0	≥1
Red/processed meat, servings/d	≥1.5	0
Fish, g/d	0	≥32.4
PUFA, % of energy	≤2	≥10
Sodium, mg/d		
Women	≥3337	≤1112
Men	≥5271	≤1612
Alcohol, drinks/d		
Women	≥2.5	0.5–1.5
Men	≥3.5	0.5–2.0
Total score	0	100

Adapted from [9]. Abbreviations: SSB: sugar-sweetened beverages; PUFA: polyunsaturated fatty acids.

Table 2. Criteria for the definition of the Mediterranean Diet Score [10].

Component	Criteria for Maximum Score (1)	
	Women	Men
Vegetables	≥181.50 g	≥148.50 g
Legumes	≥5.30 g	≥4.25 g
Fruits and nuts	≥161.15 g	≥156.85 g
Cereals	≥246.05 g	≥311.35 g
Fish	≥14.50 g	≥18.45 g
Meat	<85.15 g	<140.05 g
Dairy products	<199.45 g	<152.35 g
Alcohol	5–25 g	10–50 g
Fat intake (MUFA:SFA)	>0.75	>0.80

Abbreviations: MUFA: monounsaturated fatty acids, SFA: saturated fatty acids.

2.5. Outcome

For participants without a diagnosis of diabetes mellitus, glucose tolerance status was determined following the criteria published by the American Diabetes Association [42] based on an OGTT. Criteria that defined normal glucose tolerance status were a fasting glucose concentration of less than 5.6 mmol/L or a 2-h OGTT concentration of less than 7.8 mmol/L. Impaired glucose tolerance (2-h OGTT concentration of 7.8–11.0 mmol/L) and/or impaired fasting glucose (fasting glucose concentration of 5.6–6.9 mmol/L) was classified as prediabetes. Those participants with a fasting glucose concentration of at least 7.0 mmol/L or a 2-h OGTT concentration of at least 11.1 mmol/L were categorized under undetected diabetes mellitus. Participants with prevalent diabetes mellitus were identified by a self-reported diagnosis of T2DM or by the use of anti-diabetic medication. Prevalent cases were validated by contacting the participants' physicians. For the analysis, participants with prevalent and undetected diabetes mellitus were grouped as "T2DM participants" and participants with prediabetes and those with normal glucose tolerance status were grouped as "non-diabetic participants".

2.6. Statistical Analysis

The characteristics of the KORA FF4 participants are described for the total study population and stratified by metabolically-homogenous clusters. To assess associations between T2DM (dependent variable) and diet quality scores (independent variables), we fitted three different logistic regression models, where the AHEI was scaled by 10 points and the MDS was scaled by 2 points. Model 1 was adjusted for age, sex, and energy intake. Model 2 incorporated additional adjustment for education, physical activity, and smoking. Finally, Model 3 extended Model 2 by including waist circumference and hypertension status. All models were fitted to the total data set as well as stratified by metabotypes. Models fitted to the total data set were presented with and without further adjustment for metabotype. Additionally, for models fitted in the metabotype 1 cluster, smoking status was incorporated as a binary covariate (i.e., never smoker vs. current and former smokers) due to the limited sample size in this cluster. We report odds ratios (OR) and 95% confidence intervals (CI). Additionally, the interaction effects between metabotype and both diet quality scores were assessed by performing Likelihood-Ratio tests comparing the models, including the interaction between metabotype and diet quality scores with the models without interaction.

All statistical analyses were carried out in R software (version 3.5.3 for Windows, R Foundation for Statistical Computing, Vienna, Austria.). The significance level was set to 0.05 for all results.

3. Results

The characteristics of the study sample are described in Table 3 in total and stratified by the three metabotypes. In the total study sample, there were slightly more women (53.4%) than men and the mean age was 58.3 ± 11.6 years. Subjects in cluster 3 were more often men (64.1%), had the highest mean age (62.0 ± 11.1 years), highest BMI (32.7 ± 5.7 kg/m^2), the lowest proportion of current smokers (11.4%) and the highest proportion of prevalent diabetic participants (30.0%) compared to the other two metabotype clusters. Furthermore, metabotype cluster 3 was characterized by the lowest proportion of physically-active participants (43.6%) and the highest proportion of hypertensive participants (65%). Regarding dietary intake, these participants showed the highest mean consumption of meat and meat products (140.1 g/day) and the lowest mean consumption of vegetables (158.1 g/day). Subjects in cluster 1 appeared to be the healthiest and show the highest proportion of normal-weight participants, the highest proportion of physical active status, the lowest proportion of hypertension and the highest proportion of normal glucose tolerance status. Characteristics of participants in cluster 2 were intermediate to cluster 1 and cluster 3. There was little variation in the mean of the two diet quality scores between the metabotype clusters and both measures were in very good accordance, even though they incorporate some different food components. A detailed characterization of the metabotype clusters with respect to the clinical and anthropometric markers for the FF4 study is given in the Supplementary material of Riedl et al. [33].

Results from the logistic regression analyses are presented in Table 4. Without including metabotype information, significant inverse associations between T2DM and both scores were found in Models 1 and 2 for the total study sample. For both diet scores, an additional adjustment for education, physical activity, and smoking led to slightly higher OR estimates that are still below 1. The AHEI predicted a 36% reduction of T2DM odds compared to a 26% reduction predicted by the MDS. When additionally including the metabotype variable as adjusting factor in the total model, a significant inverse association of T2DM and the diet quality scores was only observed when the basic adjustment (age, sex, and energy intake) was applied. Remarkably, a significant interaction effect between diet score and metabotype was found in Model 2 (both scores) and in Model 3 (AHEI). To further examine these interaction effects, we conducted a stratified analysis. In the analysis stratified by metabotype, we found significant inverse diet-diabetes associations in cluster 3 only. It is noteworthy that these associations persisted with further adjustment. A significant 40% reduction of T2DM odds was observed for every 10-point increase in AHEI in Model 2. Likewise, a significant 38% reduction of the odds of diabetes mellitus was observed for every 2-point increase of the MDS in

Model 2. When comparing the different Models to each other, we observed that further adjustment only slightly affected OR estimates. No significant associations with T2DM were seen for the diet quality scores within metabotype cluster 1 or cluster 2.

Table 3. Characteristics of the study population overall and by metabotype.

	Overall (*n* = 1287)		**Cluster 1 (*n* = 591)**		**Cluster 2 (*n* = 476)**		**Cluster 3 (*n* = 220)**	
Sex, *n* (%)								
Male	600	(46.6)	182	(30.8)	277	(58.2)	141	(64.1)
Female	687	(53.4)	409	(69.2)	199	(41.8)	79	(35.9)
Age (years)	58.4	(11.6)	55.9	(11.8)	59.7	(11.1)	62	(11.1)
Education, *n* (%)								
< 10 years	67	(5.2)	23	(3.9)	29	(6.1)	15	(6.8)
10–12 years	736	(57.2)	313	(53.0)	284	(59.7)	139	(63.2)
≥ 13 years	484	(37.6)	255	(43.1)	163	(34.2)	66	(30.0)
BMI (kg/m^2)	27.5	(4.9)	24.9	(3.5)	28.3	(3.7)	32.7	(5.7)
BMI categorized, *n* (%)								
Underweight	6	(0.5)	6	(1.0)	0	(0.0)	0	(0.0)
Normal weight	422	(32.8)	323	(54.7)	89	(18.7)	10	(4.5)
Overweight	526	(40.9)	216	(36.5)	244	(51.3)	66	(30.0)
Obese	333	(25.9)	46	(7.8)	143	(30.0)	144	(65.5)
Waist circumference (cm)	95.6	(14.2)	87.4	(11.5)	98.9	(10.1)	110.9	(13.1)
Physical activity, *n* (%)								
Inactive	488	(37.9)	183	(31.0)	181	(38.0)	124	(56.4)
Active	799	(62.1)	408	(69.0)	295	(62.0)	96	(43.6)
Smoking status, *n* (%)								
Never	553	(43.0)	265	(44.8)	202	(42.4)	86	(39.1)
Current	182	(14.1)	86	(14.6)	71	(14.9)	25	(11.4)
Former	552	(42.9)	240	(40.6)	203	(42.6)	109	(49.5)
Hypertension, *n* (%)								
No	833	(64.7)	459	(77.7)	297	(62.4)	77	(35.0)
Yes	454	(35.3)	132	(22.3)	179	(37.6)	143	(65.0)
Glucose tolerance status, *n* (%)								
Normal glucose tolerance	689	(53.5)	429	(72.6)	233	(48.9)	27	(12.3)
Prediabetes	453	(35.2)	137	(23.2)	211	(44.3)	105	(47.7)
Undetected diabetes	48	(3.7)	7	(1.2)	19	(4.0)	22	(10.0)
Prevalent diabetes	97	(7.5)	18	(3.0)	13	(2.7)	66	(30.0)
Usual intake (g/day)								
Vegetables	175.9	(60.1)	190.2	(66.5)	166.5	(51.0)	158.1	(50.8)
Fruits and nuts	167.5	(85.7)	172.8	(83.4)	162.8	(86.8)	163.5	(89.0)
Meat and meat products	115.9	(43.9)	102.1	(37.0)	121.9	(43.0)	140.1	(49.4)
Fish and Shellfish	20.5	(13.1)	20.2	(13.7)	20.4	(11.8)	21.3	(14.0)
Diet quality scores								
Alternate Healthy Eating Index	44.4	(8.8)	46.1	(8.9)	43.6	(8.3)	41.5	(8.6)
Mediterranean Diet Score	4.3	(1.8)	4.5	(1.9)	4.3	(1.8)	4.1	(1.7)

Continuous variables: mean (SD), categorical variables: n (%). BMI categorized following the WHO definition (underweight: BMI < 18.5 kg/m^2, normal weight: 18.5 ≤ BMI < 25.0 kg/m^2, overweight: 25.0 ≤ BMI < 30 kg/m^2, obese: BMI ≥ 30 kg/m^2) [43]. BMI: body mass index, SD: standard deviation.

Table 4. Associations Between Diet and T2DM in the Total Sample, Stratified by Metabotype.

	Type-2 Diabetes Mellitus											
	Total (n = 1287)		Total (n = 1287) [d]			Cluster 1 (n = 591) [e]		Cluster 2 (n = 476)		Cluster 3 (n = 220)	
T2DM cases	n = 145		n = 145			n = 25		n = 32		n = 88	
	OR	95% CI	OR	95% CI	p-interaction [f]	OR	95% CI	OR	95% CI	OR	95% CI
AHEI											
Model 1 [a]	**0.59**	**0.46–0.75**	**0.75**	**0.57–0.97**	**0.031**	1.47	0.82–2.62	0.71	0.47–1.18	**0.61**	**0.41–0.90**
Model 2 [b]	**0.64**	**0.49–0.83**	0.79	0.60–1.04	**0.022**	1.52	0.83–2.78	0.83	0.49–1.41	**0.60**	**0.39–0.90**
Model 3 [c]	0.87	0.66–1.13	0.87	0.65–1.15	**0.035**	1.73	0.93–3.23	0.95	0.55–1.63	**0.60**	**0.39–0.92**
MDS											
Model 1 [a]	**0.66**	**0.52–0.83**	**0.72**	**0.55–0.94**	**0.038**	1.29	0.75–2.21	0.61	0.37–1.01	**0.59**	**0.39–0.90**
Model 2 [b]	**0.74**	**0.57–0.96**	0.78	0.58–1.03	**0.029**	1.40	0.76–2.55	0.75	0.43–1.29	**0.62**	**0.40–0.96**
Model 3 [c]	0.85	0.65–1.11	0.82	0.61–1.09	0.056	1.49	0.80–2.79	0.78	0.45–1.35	0.65	0.41–1.03

[a]: Adjusted for age, sex, energy intake. [b]: Adjusted for age, sex, energy intake, education, physical activity, smoking. [c]: Adjusted for age, sex, energy intake, education, physical activity, smoking, waist circumference, hypertension. [d]: Further adjusted for metabotypes. [e]: Given the low frequency of current smokers, logistic regressions in cluster 1 were adjusted for smoking status as never vs. former/current, [f]: Interaction between metabotype and diet quality score. Mediterranean Diet Score (MDS): models per 2 points increase in the MDS score; Alternate Healthy Eating Index (AHEI): models per 10 points increase in the AHEI score. Significant associations are printed in bold.

4. Discussion

We investigated the association between T2DM and two diet quality scores while taking into account the role of metabotypes in the population-based KORA FF4 study. We found significant interaction effects between metabotype information and both diet quality scores. In the analysis stratified by metabotype, significant inverse associations between T2DM and the two diet quality scores were only detected in the metabolically-unfavorable cluster. We observed a reduction in the odds of T2DM of about 40% for both diet scores in cluster 3. No significant associations were identified within metabotype clusters 1 and 2, i.e., the more metabolically-favorable metabotypes. Without accounting for metabotype information, significant negative associations between T2DM and both quality scores were observed in the total sample. The reduction of the odds for T2DM in the total sample was lower for both diet scores (36% and 24%) in comparison to the estimates derived from the stratified analysis in cluster 3.

When additionally including waist circumference and hypertension as adjustment variables in the logistic regression models, the association between T2DM and diet quality scores was attenuated. This might be explained by the fact that these two factors are potential mediators of diet and T2DM association (e.g., References [16,44–46]). It is known that mediating factors should not be included as adjusting factors in regression models. Therefore, our Model 3 was potentially over-adjusted. The cross-sectional design of the KORA FF4 study does, however, not allow for examining the potential mediation effect of the above-mentioned variables. The inverse association between the diet scores and T2DM was significant after adjustment for energy intake and physical activity. This supports the idea that diet quality is an independent predictor of T2DM status, independent of calorie deficit.

Following a Mediterranean diet has been shown to have protective effects on the development of T2DM by increasing insulin sensitivity and decreasing oxidative stress and inflammation [47]. Anti-inflammatory mechanisms are linked to n-3 fatty acids, which are typically consumed in larger quantities on a Mediterranean diet high in fish, legumes, and nuts [48–51]. Consumption of antioxidants also has positive effects on beta cell function and insulin resistance [52]. The consumption of these dietary components is assessed by the AHEI analogously.

It has been shown that a high-quality diet, as measured by the AHEI and the MDS indices, is inversely associated with the risk for morbidity and mortality of the major chronic diseases, namely CVD, cancer and T2DM [12,14,15,17,53–64]. We examined both indices as they have different features, although they appear similar with respect to the majority of components used. In contrast to fixed cut-off values as used by the AHEI, the MDS applies sex-specific medians of the population that is studied. Therefore, a comparison between different populations or the examination of changes over time is not an appropriate use of the MDS. On the other hand, the MDS approach ensures an equal

contribution of all components to the total score [7]. In our analyses, we obtained similar results for both diet scores. This consistency strengthens our findings.

4.1. A Priori Dietary Patterns and T2DM

Without including metabotype information in the logistic regression models, we detected significant associations of T2DM and both diet quality scores in the total sample (OR = 0.64, 95% CI = 0.49–0.83 for AHEI, OR = 0.74, 95% CI = 0.57–0.96 for MDS). Several other studies have examined the association between AHEI and T2DM. The results are mostly in line with our findings of significant associations between T2DM and diet quality scores. The Health Professionals Follow-Up Study showed a reduced risk of T2DM with increasing AHEI and increasing alternate MDS (analogous to the MDS but no dairy intake included) in American men [12]. The absolute risk reduction was accentuated among overweight and obese participants [12]. In postmenopausal women aged 50–79 years participating in the Women's Health Initiative, the AHEI and the alternate MDS were inversely associated with T2DM (multivariable-adjusted hazard ratios 0.87 and 0.90 per 1-SD increase in score, respectively) [15]. In the Multiethnic Cohort Study with participants aged 45–75 years, the AHEI and the alternate MDS, among other indices, were examined with respect to their association with T2DM. Both diet quality scores were inversely associated with T2DM [17]. The direction of the associations was mostly consistent across all ethnic groups [17]. The AHEI has been shown to be inversely associated with the risk of having T2DM in an Asian population aged 45–74 years (multivariable-adjusted hazard ratio 0.93 per 1-SD increase in score) [19]. A stratified analysis revealed a significant interaction effect of smoking on the association which was only present in non-smokers (p-interaction 0.03) [19]. This holds for the alternate MDS as well (multivariable-adjusted hazard ratio of 0.93 per 1-SD increase in score).

In American men and women from three prospective cohort studies (Nurses' Health Study (NHS), NHS II and Health Professionals Follow-Up Study) that were analyzed jointly, an improvement of diet and a worsening of diet as assessed by a change in AHEI score have been shown to be associated with a lower and higher risk of T2DM, respectively [16]. Recently, the Atherosclerosis Risk in Communities (ARIC) study did not confirm an association of AHEI and the risk of diabetes mellitus in black and white American men and women aged 45–64 years [60]. However, a significant trend of risk reduction for CVD was observed across AHEI quintiles [60].

Based on 6-year follow-up data from the Multi-Ethnic Study of Atherosclerosis (MESA), no significant association between the MDS and risk of incident diabetes mellitus was found in men and women aged 45–84 years at baseline [11]. In the EPIC-Potsdam Cohort (men and women aged 35–64 years), the MDS was significantly inversely associated with risk of diabetes mellitus (hazard ratio 0.93 per 1 SD increase in score) [18]. Earlier, an analysis of data from 8 EPIC cohorts showed a significant inverse association of the relative MDS (including the same components of the original MDS but using tertiles, not the median) and risk of T2DM [44]. The results of systematic reviews and meta-analyses confirm a relevant risk reduction of diabetes mellitus when following a Mediterranean diet as assessed by the MDS [13,14].

4.2. Inclusion of Metabotype Information to Identify High-Risk Strata

The inclusion of metabotype information when investigating the association of dietary patterns and T2DM makes our study unique. We only found significant associations between diet quality and T2DM in the cluster representing a metabolically unfavorable metabotype. Numerous cohort studies examined the effect of following certain dietary patterns on the risk of incident T2DM. In most studies, inverse associations were revealed, offering a promising possibility to implement prevention actions for populations based on such dietary patterns. These approaches, however, do not take into account other factors, either intrinsic or extrinsic, that influence the response or non-response to a dietary pattern. Important examples include the composition of gut microbiota and responsiveness to a Nordic diet, as discussed in a review paper [65], the relationship of weight loss and insulin and glucose status [66], or vitamin D responsiveness and markers of metabolic syndrome [67]. Our analysis

included metabotype information as a novel feature when analyzing the association between T2DM and dietary patterns. Metabotypes describing metabolically-similar individuals have previously been suggested [31] and successfully applied in the field of tailored dietary advice [35]. Therefore, we expected to see differences in the associations of T2DM and dietary patterns across the metabotypes. Only in cluster 3, the metabolically least-favorable cluster, were significant inverse associations between T2DM and the diet quality scores present. Prospective studies including metabotype information are needed to further elucidate a possible causal relationship. This could confirm the hypothesis that the protective effect of diet on T2DM is modified by underlying metabotypes, i.e., by metabolic characteristics. This would allow for the identification of metabolically-homogenous subgroups that would benefit the most from the improvement of the quality of the diet as measured by AHEI and MDS.

4.3. Strength and Limitations

The KORA FF4 study is the second follow-up of KORA S4, a population-based study in the Augsburg study region. The KORA S4 survey was characterized by a high participation rate and was representative of the general Bavarian population. The assessment of glucose tolerance status was carried out by trained and experienced staff and has been validated. The dietary assessment was extensive, and the usual intake was derived by applying a sophisticated method. The estimation of usual intake is based on combining information obtained by long-term and short-term dietary assessment instruments. The application of two diet quality scores and the subsequent results obtained in our analyses strengthen our findings. Nonetheless, the number of prevalent diabetic or unknown diabetic participants is rather small. Due to the limited sample size, some categories for glucose tolerance status had to be combined for the analysis. This resulted in a loss of detailed information. A further limitation of our study is its cross-sectional nature. Our analysis does not allow for drawing causal conclusions about the association of T2DM and a priori dietary patterns. Still, we assume that including undiagnosed diabetic patients in the group of cases strengthens our findings. These participants were not aware of the diagnosis and therefore did not make healthy changes to their dietary behavior, as was probably the case for the already diagnosed participants. Regarding the dietary assessment, we cannot rule out recall bias or over- or under-reporting. One major limitation of the metabotype concept is that there is still no unique definition of the term "metabotype" available, nor a single way to identify homogenous groups, as reviewed in Reference [32] and recently in Reference [68], with a focus on nutrition.

5. Conclusions

We investigated the role of metabotypes in the association of dietary patterns and T2DM. The inverse association between AHEI and MDS and T2DM was only present among those with an unfavorable metabotype. Prospective studies including metabotype information are needed to confirm these results, which allow for the tailoring of dietary recommendations in the prevention of T2DM.

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5-*cis*-, *Trans*- and Total Lycopene Plasma Concentrations Inversely Relate to Atherosclerotic Plaque Burden in Newly Diagnosed Type 2 Diabetes Subjects

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Abstract: Diabetic subjects are at increased risk of cardiovascular disease. Atherosclerosis, the common soil of most of the cardiovascular complications, is more prevalent and extensive in this population due not only to hyperglycemia, insulin resistance, and dyslipidemia, but also to inflammation and oxidative stress. Lycopenes are bioactive compounds with antioxidant and anti-inflammatory activities mostly supplied by tomato and tomato byproducts. We investigated the association between circulating lycopenes and carotid plaque burden in diabetic patients, in a cross-sectional study in 105 newly diagnosed diabetic subjects. Atheroma plaque (wall thickness ≥ 1.5 mm), number of plaques, and plaque burden (sum of maximum heights of all plaques) were assessed by sonographic evaluation of carotid arteries. Plasma lycopenes (5-*cis*-, 9-*cis*-, 13-*cis*-, and *trans*-lycopene) were quantified by high performance liquid chromatography–mass spectrometry HPLC-MS. Atheroma plaque was observed in 75 participants, from which 38 presented one plaque and 37 two or more carotid plaques. No differences were observed in the plasmatic concentrations of lycopenes between subjects with and without atherosclerotic plaque presence. However, plaque burden was inversely associated with 5-*cis*-lycopene, all *cis*-lycopene isomers, *trans*-lycopene, and total lycopene isomers (all, $p < 0.05$). High plasma levels of lycopenes inversely relate to atherosclerotic burden. We provide novel evidence that suggests that the consumption of compounds found in tomato and tomato byproducts might be beneficial for the prevention of atherosclerosis.

Keywords: atherosclerosis; plaque burden; lycopene; tomato; type 2 diabetes mellitus; *cis*- and *trans*-isomers

1. Introduction

The progressive westernization of dietary patterns coupled with physical inactivity has caused a significant increase in the prevalence of obesity and a global epidemic of type 2 diabetes mellitus, expecting to affect 642 million people by 2040 [1]. The first cause of blindness, nontraumatic amputation, and terminal kidney disease is microvascular complications of diabetes, and cardiovascular disease is the first cause of mortality in this population [2]. Therefore, preventing and/or delaying the progression of cardiovascular complications in diabetes is a major public health concern.

Atheroma plaque is the hallmark of most of the cardiovascular events. In diabetes, hyperglycemia, insulin resistance and dyslipidemia lead to a proinflammatory and prooxidant status which promotes accelerated atherogenesis. In fact, atherosclerosis begins earlier, is more extensive, and progresses more rapidly than in nondiabetic individuals [3]. Together, this prompted the notion that dietary consumption of bioactive compounds with antioxidant and anti-inflammatory activities might contribute to the protection against atherosclerosis progression.

Lycopene is a red fat-soluble pigment widely found in vegetables and fruits, but more specifically in tomato and tomato byproducts. The term "lycopene" comprises different isomers suggested to differ in their origin, bioavailability and presumed healthy effects [4]. Several epidemiologic studies have evaluated the relationship between lycopene intake (without distinction among isomers), evaluated either by food frequency questionnaires [5–7] or serum/plasma concentrations [5–12], and ultrasound-assessed subclinical atherosclerosis with somewhat controversial results. Null [5,7,9] or inverse [6,8,10–12] associations have been reported between lycopene and common carotid artery intima media thickness (CCA-IMT), a very early and widely used surrogate marker of cardiovascular risk [12]. However, carotid plaque burden (defined as sum of plaque heights or plaque areas), rather than IMT, is more representative of atherosclerosis and is a better predictor of cardiovascular events [13].

To date, studies relating dietary lycopene and carotid atheroma plaque are scant [6], and none of them has been performed in a diabetic population. We therefore hypothesized that circulating lycopene would inversely relate to carotid plaque burden in diabetic patients. To address this issue, we aimed at investigating the association between lycopene consumption and subclinical carotid atheroma plaque and burden in a group of newly diagnosed diabetic patients.

2. Materials and Methods

2.1. Study Population

In this cross-sectional study, we included 105 diabetic subjects from the DIABIMCAP Study (Carotid Atherosclerosis in Newly Diagnosed Type 2 Diabetic Individuals, ClinicalTrials.gov Identifier: NCT01898572), which aims at investigating preclinical atherosclerosis in new-onset type 2 diabetes mellitus subjects [14]. The institutional ethics committee approved the protocol of the study, and it was performed in accordance with the Declaration of Helsinki. All subjects included in the study underwent a physical examination in a first visit at their primary health care center, and were selected according to inclusion and exclusion criteria. After acceptance of inclusion in the study, they signed an informed consent. Inclusion criteria were clinical and laboratory evidence of type 2 diabetes (lack of autoimmune diabetes or antiglutamic acid decarboxylase negativity in suspicious cases; and/or fasting glucose and/or HbA1c, 1999 WHO criteria, respectively), and within one year of evolution prior to inclusion in the study. Exclusion criteria were as follows: prior history of cardiovascular disease or congestive heart failure; chronic diseases such as cancer, renal failure, liver disease, or other debilitating disease; short life expectancy; history of chronic alcohol or drug abuse or dependence; and major psychiatric disease [14].

2.2. Clinical and Laboratory Determinations

At inclusion, age, sex, clinical, sociodemographic and anthropometric data were recorded. An Omron HEM-7223-E (Hoofddorp, The Netherlands) was used to determine blood pressure the

same day of carotid ultrasound performance. Considering the fact that a high percentage of individuals were under statin treatment, known to modulate plaque regression, we calculated a statin score for statistical adjustment. The statin score was calculated as the years of cholesterol-lowering treatment multiplied by the dose of these drugs (average) normalized to simvastatin, and pretends to estimate lifelong exposure to hypolipidemic treatment.

Fasting blood samples were collected in tubes with the anti-coagulant ethylenediaminetetraacetic acid (EDTA) or serum tubes. Blood was centrifuged at 3000 rpm for 10 min at 4 °C, within 30 min of venipuncture, and plasma and serum were stored at −80 °C until analyses. Biochemical measurements were performed in the Biomedical Diagnostic Center, Hospital Clinic, Barcelona, Spain. Total cholesterol and triglycerides were measured using the COD-PAP and GPO-PAP method (cholesterol oxidase/glycerol phosphate oxidase coupled to phenol and 4-aminophenazone). High density lipoprotein (HDL) cholesterol and high sensitivity C reactive protein (hsCRP) were measured using commercially available kits by a polyethylene glycol enhanced immunoturbidimetric assay (Siemens AG, Erfurt, Germany). Low density lipoprotein (LDL) cholesterol was estimated with the Friedewald equation when patients were fulfilling the criterion of triglycerides lower than 400 mg/dL. Glucose was measured by the hexokinase method (ADVIA 2400 Chemistry Systems analyzer, Siemens AG, Erfurt, Germany).

Lipoprotein analysis in serum was performed by two-dimensional (2D) diffusion-ordered proton nuclear magnetic resonance (1H NMR) spectroscopy (DOSY), as reported [15]. In brief, 2D 1H NMR spectra were recorded on a BrukerAvance III 600 spectrometer at 310 K (Bruker BioSpin, Rheinstetten, Germany). Double stimulated echo pulse program with bipolar gradient pulses and a longitudinal eddy-current delay was performed. The relaxation delay was 2 s, the finite impulse decays were collected into 64 K complex data points and 32 scans were acquired per sample. The gradient pulse strength was increased from 5% to 95% of the maximum strength of 53.5 Gauss cm1 in 32 steps. The squared gradient pulse strength was linearly distributed. Data of lipid concentrations, very low-density lipoprotein (VLDL), LDL and HDL size and number of each type of particle, as well as subtypes of particles according to their sizes (large, medium and small) were obtained.

The extraction and isolation of lycopene isomers from human plasma was carried out avoiding light exposure, as described in Arranz et al. [16]. Briefly, plasma was extracted twice with hexane, and the organic phases were isolated, pooled, and evaporated under nitrogen flow. Finally, the residue was reconstituted with methyl tert-butyl ether (MTBE) up to 1 mL and filtered through a 13 mm, 0.45 µm polytetrafluoroethylene filter (Waters, Milford, MA, USA) into an insert-amber vial for chromatographic analysis. Samples were stored at −80 °C until analysis. The analytes of interest, namely 5-*cis*-, 9-*cis*-, 13-*cis*-, and *trans*-lycopene, were separated and identified by high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) according to the procedure described by Vallverdu-Queralt et al. [17], using an HP 1100 high-performance liquid chromatography system (Hewlett–Packard, Waldbronn, Germany) coupled to an API 3000 triple-quadrupole mass spectrometer (Sciex, Framingham, MA, USA) equipped with a turbo ion spray source in positive ion mode. Results are expressed as µmol/L of plasma.

2.3. Sonographic Assessment of Carotid Atherosclerotic Plaques

IMT and atherosclerotic plaque presence were evaluated through standardized bilateral carotid artery ultrasound imaging with an Acuson X300 ultrasound system (Siemens AG, Erfurt, Germany) equipped with a VF 10–5 linear transducer (5 to 10 MHz frequency range) [14], in the second visit. The same certified sonographer and certified reader performed all determinations, and these were quantified off-line with a semiautomatic validated software [18]. Plaques were analyzed by B-mode and color Doppler, and defined as a focal wall thickening encroaching into the arterial lumen by at least 50% of the surrounding IMT value or with thickness of at least 1.5 mm as measured from the media adventitia interference to the intima-lumen surface [19]. Plaque height was recorded either longitudinal or transversal, as appropriate. Plaque burden was calculated as the sum of maximum heights of all plaques. Variability of ultrasound carotid wall measurements was quantified by repeating

3 different days the determinations from 14 participants. Intraclass correlation coefficient was 0.92–0.96 for mean and maximum IMT (average of right and left, and maximum value from either right or left, respectively) in common, bulb, and internal carotid segments.

2.4. Dietary Intake

Assessment of dietary intake was performed with the 137-item semiquantitative food-frequency questionnaire validated for the PREDIMED study [20]. Subjects were asked, in an in-person interview, about dietary habits within the last year through the number and size of serving of each food item, ranging from never or almost never to >6 times a day. This questionnaire was processed with the Food Processor Nutrition and Fitness Software (ESHA Research, Salem, OR 97306, United States) to obtain dietary data. Information on tomato-based products was collected in 3 items of the questionnaire (raw tomato; "gazpacho" (a cold Spanish soup made from tomatoes, peppers, and other salad vegetables); and ketchup/fried tomato sauce).

2.5. Clinical and Laboratory Determinations

Normal distribution of data was assessed using graphical methods and the Shapiro–Wilk test. Variables with skewed distribution were processed by a logarithmic transformation prior to statistical analysis and are presented as antilogarithms to facilitate interpretation of the results. Descriptive data are expressed as means ± standard deviations or as absolute frequencies and percentages (categorical variables). Spearman's correlation coefficient was used to study the association of plasmatic concentrations of the different lycopene species (and the sum of them as well) with dietary intake of tomato-based foods, cardiovascular risk factors, and with plaque burden. Associations between carotid atherosclerosis and circulating lycopenes were assessed by analysis of variance (ANOVA) and the Bonferroni post hoc test, and by constructing linear (plaque number; plaque burden) or logistic (plaque presence) regression models. The models were adjusted for age, sex, BMI, treatment with antihypertensive agents, smoking and statin score as potential confounders. Statistical significance was set at the $p < 0.05$ level in all cases. Analyses were performed using SPSS software, version 19.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Participants' Characteristics

Table 1 shows the clinical characteristics of the 105 participants with new-onset type 2 diabetes mellitus included in the study. Atheroma plaque was observed in 75 (71%) participants. No differences were observed in sex, age, anthropometric or biochemical variables between subjects with and without atherosclerosis.

3.2. Dietary Intake

According to dietary questionnaires, no differences were observed in nutrient intake between subjects with and without atherosclerosis (Table S1). In addition, no differences were observed in tomato consumption according to the main items of the questionnaire of frequency of consumption (Table S2). Self-reported dietary consumption of tomato and tomato byproducts was directly associated to *trans*-lycopene. Furthermore, heat-treated tomato byproducts were directly associated with 5-*cis*-lycopene ($p = 0.032$) and 9-*cis*-lycopene ($p = 0.049$), and, limited to a trend with 13-*cis*-lycopene ($p = 0.078$), as can be observed in Table 2.

Table 1. Clinical characteristics of the 105 participants with diabetes included in the study.

Variable	Subjects without Atherosclerosis (n = 30)	Atherosclerotic Subjects (n = 75)	p	Reference Interval [1]
Females, n (%)	15 (50)	33 (44)	0.577	-
Age, years	58.7 ± 8.38	61.23 ± 6.83	0.112	-
BMI (kg/m^2)	31.52 ± 5.49	30.75 ± 4.98	0.489	20–24.9
Waist (cm)	105.02 ± 11.47	104.16 ± 13.34	0.765	<100 (women) <110 (men)
Systolic blood pressure (mmHg)	129.13 ± 17.38	132.57 ± 16.46	0.343	<140
Diastolic blood pressure (mmHg)	83.17 ± 10.74	81.93 ± 9.73	0.570	<90
Glucose (mg/dL)	139.17 ± 16.41	140.18 ± 40.59	0.857	65–110
Insulin (UI/L)	20.67 ± 16.67	18.84 ± 12.15	0.538	2–15
HOMA-IR	7.26 ± 6.33	6.56 ± 4.45	0.529	<2.15
HbA1C (%)	6.92 ± 1.43	7.41 ± 1.87	0.156	3.4–5.5
hsCRP (mg/L)	0.59 ± 0.69	0.51 ± 0.53	0.488	<1.07
Triglycerides (mg/dL)	144.9 ± 94.66	151.38 ± 84.29	0.733	50–150
Total cholesterol (mg/dL)	198.83 ± 32.47	200.5 ± 44.48	0.833	148–200
HDL (mg/dL)	50.32 ± 14.84	49.09 ± 13.89	0.701	>50 (women) >40 (men)
LDL (mg/dL)	165.98 ± 50.71	174.54 ± 56.03	0.486	<130
ApoA1(mg/dL)	141.46 ± 25.02	138.01 ± 21.16	0.488	102–215
ApoB (mg/dL)	95.96 ± 23.94	104.74 ± 27.85	0.145	59–155
nonHDL (mg/dL)	146.83 ± 30.52	151.18 ± 41.87	0.559	<100
Hypertension, n (%)	15 (30)	41 (55)	0.665	
Dyslipidemia, n (%)	11 (37)	32 (43)	0.572	
Smokers, n (%)	4 (13)	16 (21)	0.346	
History of premature CVD, n (%)	2 (7)	8 (11)	0.254	
Medication, n (%)				
GLP-1 agonists	11 (37)	41 (55)	0.096	
Pioglitazone	11 (37)	41 (55)	0.096	
Statin score [2]	16.02 ± 34.9	23.36 ± 46.08	0.433	

Data are expressed as mean ± SD, except for categorical variables, expressed as n (%). p from the comparison between subjects with and without carotid atherosclerosis (t-test for quantitative and chi square test for qualitative variables). [1] Reference values obtained from the core laboratory of the Hospital Clínic of Barcelona, Spain. [2] Statin score was calculated as the product of the duration of treatment in years by the average dose received of statin drugs standardized to simvastatin. BMI indicates body mass index; HOMA-IR, homeostatic model assessment for insulin resistance; hsCRP, high sensitivity C-reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein; CVD, cardiovascular disease; and GLP-1 denotes glucagon-like peptide-1.

Table 2. Correlations between self-reported dietary intake of tomato-based food items and plasmatic concentrations of different lycopene species.

Food Item	5-*cis*-Lycopene	9-*cis*-Lycopene	13-*cis*-Lycopene	*trans*-Lycopene	Sum of Lycopenes
Raw tomato, g/day					
Rho Spearman [1]	0.189	0.190	0.154	0.184	0.209
p [1]	0.073	0.072	0.145	0.082	0.046
Gazpacho, g/day					
Rho Spearman [1]	0.179	0.166	0.139	0.185	0.194
p [1]	0.089	0.115	0.187	0.080	0.065
Ketchup/fried tomato sauce, g/day					
Rho Spearman [1]	0.227	0.210	0.188	0.215	0.206
p [1]	0.032	0.049	0.078	0.041	0.053
Total tomato-based foods, g/day					
Rho Spearman [1]	0.237	0.211	0.180	0.227	0.244
p [1]	0.025	0.047	0.091	0.031	0.021

[1] Coefficients of correlation and p values from the Spearman analyses.

3.3. Association between Lycopene Isomers and Lipoprotein Particles

As displayed in Table S3, the number of total, large and medium VLDL particles was independently associated with carotid plaque presence and burden.

No significant associations were observed between VLDL and LDL particles and lycopene isomers. However, small HDL particles were directly associated with 13-*cis*-lycopene (Spearman's Rho 0.230; $p = 0.031$), all *cis*-lycopenes (Spearman's Rho 0.292; $p = 0.015$), *trans*-lycopene (Spearman's Rho 0.240; $p = 0.049$), and total lycopene isomers (Spearman's Rho 0.266; $p = 0.028$). A trend was also observed for 5-*cis*-lycopene (Spearman's Rho 0.174; $p = 0.090$). These associations were maintained after multivariate adjustments ($p = 0.034$ for 13-*cis*-lycopene, $p = 0.040$ for 5-*cis*-lycopene, $p = 0.027$ for all *cis*-lycopene isomers, $p = 0.042$ for *trans*-lycopene, and $p = 0.030$ for total lycopene isomers).

3.4. Association between Lycopene Isomers and Atherosclerotic Burden

Among participants with atherosclerosis, one plaque was identified in 38 subjects and two or more plaques in 37 (35%) subjects. No differences were observed in the plasmatic concentrations of lycopenes between subjects with and without atherosclerotic plaque presence. However, subjects with two or more carotid plaques had significantly lower plasma concentrations of 5-*cis*-lycopene, *trans*-lycopene, and total lycopene isomers, compared to those subjects without atherosclerosis or with only one plaque (Table S4). Nonetheless, these associations disappeared after multivariate adjustment. Finally, plaque burden was inversely associated with 5-*cis*-lycopene, all *cis*-lycopene isomers, *trans*-lycopene, and total lycopene isomers, as depicted Figure 1.

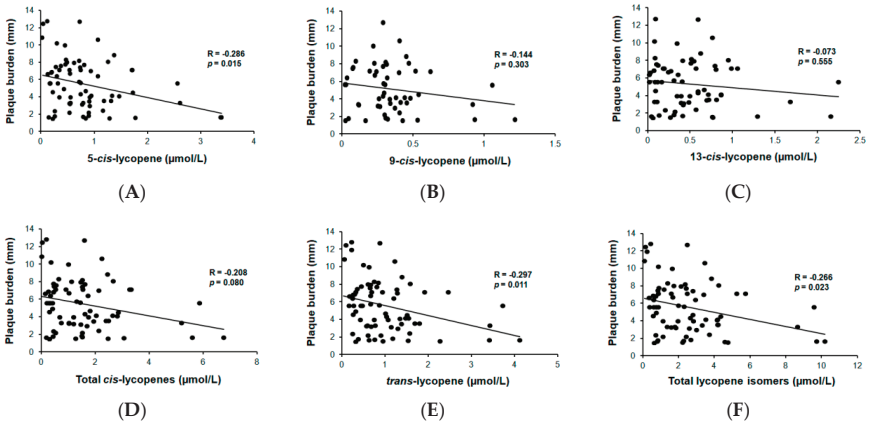

Figure 1. Correlations between plasmatic concentrations of 5-*cis*-lycopene (**A**), 9-*cis*-lycopene (**B**), 13-*cis*-lycopene (**C**), total *cis*-lycopenes (**D**), *trans*-lycopene (**E**), total lycopene isomers (**F**) and plaque burden. R (Rho coefficient) and *p* value from the Spearman's correlation analyses. Plaque burden was calculated as the sum of maximum heights of all plaques. Data from $n = 75$ participants.

These associations remained after multivariate adjustments except for all *cis*-lycopene isomers, as shown in Table 3.

Table 3. Multivariate associations of plasmatic lycopenes with carotid plaque burden in the 105 diabetic subjects included in the study.

Lycopene Measurement	B (95% CI)	*p*
5-*cis*-lycopene	−0.99 (−2.01, −0.02)	0.045
9-*cis*-lycopene	−1.26 (−4.56, 2.04)	0.445
13-*cis*-lycopene	−0.54 (−2.09, 1.01)	0.487
all *cis*-lycopene	−0.40 (−0.91, 0.11)	0.122
trans-lycopene	−0.86 (−1.71, −0.01)	0.047
Total lycopene isomers	−0.31 (−0.64, −0.01)	0.050

Data are presented as nonstandardized regression coefficient (B) for the association between each variable and plaque burden, with 95% confidence intervals, examined by multiple linear regression analysis adjusting for age, sex, body mass index, smoking habits (yes/no), hypertension, and statin score. Plaque burden was calculated as the sum of maximum heights of all plaques. Data from $n = 75$ participants.

4. Discussion

In this cross-sectional study, we examined the potential associations between plasmatic lycopene isomers and atherosclerosis in subjects with diabetes. Plasma concentrations of lycopenes were inversely related to atherosclerotic burden in these subjects, suggesting that tomato and tomato byproducts consumption, in the context of a healthy diet, may be beneficial against atherosclerosis progression.

Atherosclerosis is triggered by increased levels of cholesterol in the intima and driven by a multifactorial process including inflammation and oxidation [21]. In this setting, lycopenes have been shown to improve endothelial dysfunction by modulating the endothelial nitric oxide synthase (eNOS) pathway [3,22], and by reducing blood pressure [23]. In addition, they have been shown to induce a reduction of cytokines and adhesion molecules levels such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular Adhesion Molecule 1 (ICAM-1) [12,24], and to protect against LDL oxidation [25,26] by inhibiting the myeloperoxidase activity [3] or through inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [27]. Therefore, it seems plausible that high concentrations of circulating lycopenes, reflecting diets rich in tomato and tomato byproducts, may protect against atherosclerosis burden and progression in subjects with diabetes [28].

The associations between plasma metabolites and cardiovascular (and overall health) outcomes may be considered in the perspective of food and diet composition as a whole in spite of as the effects of a single compound or metabolite. It is known that food processing modulates the proportion of different lycopene isomers in the available foods. In this regard, both the cooking time and the presence of onions was found to increase the *cis*-isomers of lycopene in the so-called "sofrito" [29], a slow-cooked combination of tomato, olive oil, garlic and onion, widely consumed in Spain, being considered an important part of the Mediterranean Diet in this country. In fact, in healthy individuals, raw tomato consumption has been shown to decrease cellular and plasma proinflammatory biomarkers related to the onset and progression of atherosclerosis, and these effects were more pronounced after the consumption of sofrito-like cooked tomato sauce with olive oil [30], in which lycopenes are more bioavailable [17,31]. Along this line, sofrito consumption has been shown to decrease proinflammatory biomarkers such as C-reactive protein and tumor necrosis factor-alpha in healthy subjects [32] as well as in obese and overweight women [33].

Tomato consumption and, by extension, lycopene consumption, has been shown to improve the lipid profile by decreasing triglyceride and total cholesterol levels and raising HDL cholesterol in healthy individuals [30] and overweight women [34]. In type 2 diabetes subjects, tomato juice consumption decreased susceptibility of LDL to oxidation [35], and daily consumption of 200 g raw tomato for 8 weeks decreased both systolic and diastolic blood pressure and increased ApoA1 plasma concentrations [36]. We did not find any association between lycopene plasma metabolites, surrogate biomarkers of tomato consumption, and blood pressure, triglycerides, total, LDL or HDL cholesterol. Of note, we did observe a positive association between total lycopenes (and some specific isomers) and small HDL particles. Although no differences were observed in the levels of small HDL particles

in subjects with different atherosclerotic burden in our study population, it is worth mentioning that these particles are inversely related to cardiovascular mortality [37].

A meta-analysis concludes that the consumption of tomato and tomato byproducts, as well as lycopene supplementation, may prevent the development of cardiovascular disease by decreasing several risk factors [38], and thus, the consumption of tomato and tomato byproducts, as well as other lycopene-rich products may significantly impact cardiovascular disease onset and progression [39]. We did not observe significant correlations between lycopene concentrations and classical cardiovascular risk factors, probably because type 2 diabetes subjects are already at increased cardiovascular risk. However, we did observe that subjects with significant atherosclerotic burden had lower plasma concentrations of 5-*cis*-lycopene, *trans*-lycopene, and total lycopene isomers, even when adjusting for cardiovascular risk factors. Since atherosclerosis is a systemic disease, detection of atheroma plaque by noninvasive techniques in the easily accessible carotid artery emerged as a surrogate marker for advanced atherosclerosis in other vascular beds, including coronary arteries. Moreover, carotid plaque is a major contributor to stroke on its own. In accordance with our results, it has been reported that elevated levels of total lycopenes are associated with decreased risk of stroke in men [40].

Our findings might contribute to shed light on the existing controversy on whether lycopene relates to atherosclerosis, since either favorable [5,7,9] or null associations [6,8,10–12] with CCA-IMT were previously reported. A plausible explanation for such conflict, beside differences in dietary patterns between populations, might be that no differences between lycopene isomers have been taken into account. To our knowledge, previous studies have approached this question by considering lycopene as a whole [3,12,22,23,25–27] or considering *trans*- lycopene species [24], but never exploring other lycopene isomers as exposures of interest. In addition, these studies were not performed in type 2 diabetes subjects, in which the effects of lycopene-rich foods consumption may differ from those observed in populations at lower intrinsic cardiovascular risk.

Our study is not exempt of limitations. First, because of its cross-sectional design, the causal link cannot be inferred. Second, the study lacks sample size calculation. Our exploratory study was performed in a subsample of individuals with diabetes embedded within a larger cross-sectional study, and, therefore, this hypothesis regarding diabetic individuals should be confirmed by further studies powered enough to uncover associations of interest or, even better, by randomized control studies. In addition, plaque calcification was not measured and would provide additional information on the relationship between lycopene consumption and severity of atherosclerosis. Finally, since the study was conducted in subjects with new diagnosis of type 2 diabetes mellitus, our results cannot be generalized to other populations. However, as a main strength of our study, we have measured objective biomarkers of intake, which plasmatic concentrations significantly correlate to self-reported consumption of tomato-based foods.

5. Conclusions

The plasmatic concentration of *cis*-, *trans*- and total lycopene metabolites inversely relate to plaque burden in newly diagnosed type 2 diabetes subjects at high cardiovascular risk. Long-term randomized trials are required to confirm that lycopene-rich products, such as tomatoes and their derivatives, might help in preventing or at least delaying the onset and progression of atherosclerosis, and consequently major cardiovascular events.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/6/1696/s1, Table S1: Estimated nutrient intake in the 105 participants with and without atherosclerosis included in the study, Table S2: Estimated consumption of tomato and tomato byproducts in the 105 participants with and without atherosclerosis included in the study, Table S3: Lipoprotein particle number in study participants with and without atherosclerosis, and Table S4: Associations between plasmatic lycopenes with carotid plaque number in the 105 diabetic subjects included in the study.

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Acute Effects of the Consumption of *Passiflora setacea* Juice on Metabolic Risk Factors and Gene Expression Profile in Humans

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Abstract: Background: *Passiflora setacea* (PS) is a passionfruit variety of the Brazilian savannah and is a rich source of plant food bioactives with potential anti-inflammatory activity. This study aimed to investigate the effect of an acute intake of PS juice upon inflammation, metabolic parameters, and gene expression on circulating immune cells in humans. Methods: Overweight male volunteers (n = 12) were enrolled in two double-blind placebo-controlled studies. Blood samples were collected from fasting volunteers 3 h after the consumption of 250 mL of PS juice or placebo (PB). Metabolic parameters (insulin, glucose, total cholesterol, high-density lipoprotein (LDL), high-density lipoprotein (HDL), and total triglycerides) and circulating cytokines were evaluated (study 1). Peripheral blood mononuclear cell (PBMC) from the same subjects were isolated and RNA was extracted for transcriptomic analyses using microarrays (study 2). Results: Insulin and homeostatic model assessment for insulin resistance (HOMA-IR) levels decreased statistically after the PS juice intake, whereas HDL level increased significantly. Interleukin (IL)-17A level increased after placebo consumption, whereas its level remained unchanged after PS juice consumption. Nutrigenomic analyses revealed 1327 differentially expressed genes after PS consumption, with modulated genes involved in processes such as inflammation, cell adhesion, or cytokine–cytokine receptor. Conclusion: Taken together, these clinical results support the hypothesis that PS consumption may help the prevention of cardiometabolic diseases.

Keywords: *Passiflora setacea*; bioactive compounds; phenolic compounds; cardiovascular diseases; nutrigenomics; gene expression; immune system; cytokines; insulin; HDL

1. Introduction

According to the World Health Organization (WHO), noncommunicable diseases (NCDs) are responsible for 71% of deaths worldwide, leading to the death of 15 million people aged between 30 and 69 years old. The most prevalent diseases are cardiovascular diseases, followed by cancers, respiratory diseases, and diabetes [1]. At the same time, the total number of people suffering from depression or

other common mental disorders such as anxiety was estimated as exceeding 300 million people in 2015. These disorders are the biggest contributors to global disability and represent an important cost burden [2]. Therefore, stressful lifestyle markers such as emotional stress, an unhealthy diet (high in sugar, sodium, red meat, and trans fatty acids, but low intake of fruits and vegetables), overweight [3], and poor physical activity [4] increase the incidence of cardiovascular diseases (CVD) and NCDs. These lifestyle risk factors promote high blood pressure, hyperglycemia, hyperinsulinemia, hypertension, hyperlipidemia [1,5], obesity [1], high inflammatory cytokine production [6], and pro-atherogenic gene profile [7], and are associated with chronic low-grade inflammation and vascular inflammation [8].

A higher intake of fruit and vegetables is associated with a lower risk of all causes of mortality, particularly inflammation-related diseases [9]. Plant-based foods are sources of a variety of bioactive compounds (BC) such as terpenoids (carotenoids, essential oil components, phytosterols), polyphenols (flavonoids and non-flavonoids compounds) [10], sulfur compounds (glucosinolates and ally sulfinates), alkaloids [11], and polyamines [12], whose level of total intake is connected with the protection from chronic diseases, including cardiovascular diseases, cancers, and neurodegenerative diseases [11,12]. Several beneficial effects have been related to the consumption of these compounds such as antioxidant and anti-inflammatory activities [12,13]. These beneficial effects derive from the reported capacity of some BC to modulate cell signaling and consequently the expression of key genes [14]. The species of *Passiflora* genus have been studied due to their sedative, anxiolytic, anti-inflammatory, antioxidant, and anti-carcinogenic effects [15,16]. *Passiflora setacea* D.C (PS) is a wild passionfruit species of the Brazilian savannah, popularly known as "maracujá do sono" ("sleep passionfruit"). The consumption of these fruits has been traditionally associated with sleep modulation [17]. PS pulp and seeds have recently been identified as rich in BC, particularly in C-glycosides of flavonoids [18], and also homoorientin, vitexin, isovitexin, and orientin at higher contents than those found in *Passiflora edulis*, açaí (*Eurydema oleracea*), and orange juice [19]. They have also revealed antioxidant and antimicrobial properties in vitro [20,21]. These effects are potentially due to the presence of vitamin E and BC such as terpenoids, polyamines, and polyphenols, especially orientin, isoorientin, vitexin, and isovitexin [17–19,21]. These compounds have been reported to exert antioxidant, anti-inflammatory, vascular, neuroprotective, anxiolytic, and antidepressant-like effects [22–26]. Plant-based diets are recognized for their beneficial effects on the modulation of intermediate risk factors for inflammation-based disorders [27,28], and fruits constitute major contributors to these effects [29]. However, clinical studies focusing on the health properties of fruits of the Brazilian savannah, as well as on the potential underlying molecular mechanisms, including the modulation of expression of genes in humans, remain scarce. Therefore, the aim of our study was to evaluate the effect of acute consumption of PS pulp on metabolic and inflammatory biomarkers in overweight male volunteers, as well as to assess the impact on global gene expression profile in peripheral blood mononuclear cell (PBMC) by using microarray analyses.

2. Materials and Methods

2.1. Processing and Characteristics of Passiflora setacea Juice

The fruit used in this study was the PS, the BRS *Pérola do Cerrado* (BRS Pearl of the Brazilian savannah), which is cultivated at the experimental field of Embrapa Cerrados, Brasilia, Brazil, affiliated with the Brazilian Ministry of Agriculture. This study is part of a larger program called the Passitec Network, developed to improve fruit size and production. PS plants were cultivated in a vertical espalier system and the ripe fruits were harvested at their full maturity level during the rainy season, corresponding to the stage where the phenolics compounds were in their highest concentrations [19].

The pulps used in this experiment were prepared all at once and were aliquoted, thus allowing us to use the same batch of pulp throughout the study. The pulps were removed from the fruits and blended for 30 s to separate the seeds from the pulps by sieving. After that, they were aliquoted into portions of 150 g and placed into plastic bags, hermetically sealed, and stored at −80 °C. The batch of

pulp used in this study contained 2.75 g/100 g in fresh weight (FW) of carbohydrates, 10.1 mg/100 g FW of vitamin C, 55.4 mg/100 g of proanthocyanidins, 86 mg gallic acid equivalents (GAE)/100 g FW of total phenolics, and 3.02 mg quercetine equivalents (QE)/100 g FW of total flavonoids in which we have results for the four main flavone C-glycosides (1.07 mg/g dry weight (DW) of orientin, 0.99 mg/g DW of isoorientin, 0.84 mg/g DW of vitexin, 1.13 mg/g DW of isovitexin) and for the flavanone glycoside (0.14 mg g^{-1} FW of hesperetin equivalent) [19]. The isocaloric placebo drink (PB) was obtained by mixing 100 mL of a passionfruit-flavored isotonic drink of the brand Gatorade with 150 mL of water to achieve the same final volume and sugar content of the PS juice (List S1).

2.2. Subjects and Study Design

Male volunteers (n = 12) were recruited by interviews after advertisements were published in the media (newspaper, website, etc.) from February to June 2015. Men, ranging from 40 to 64 years old, who were overweight or slightly obese (based on body mass index (BMI) between 25 and 31 kg/m^2 or waist circumference >94 cm), non-smokers, and engaged in a low to moderate level (<5 h/week) of physical activity were eligible for inclusion. The exclusion criteria included a medical history of cancer or severe metabolic diseases, special dietary habits (e.g., vegetarians and vegans), use of dietary supplementation 2 months prior to the experiment (vitamin C, multivitamin, antioxidant capsules, etc.), chronic medication (anti-hypertensives, anti-hyperglycemic, anti-cholesterol, anti-depressants, anxiolytics, etc.), acute treatments 15 days prior to the experiment (anti-inflammatory drugs, antibiotics, etc.), and acute treatments 2 days prior to the experiment (inflammatory pain relievers such as aspirin, acetaminophen, etc.). A physical evaluation was performed to obtain measurements of weight, BMI, waist circumference, and percentage of body weight by applying the seven skinfold sites Jackson–Pollock method [30].

The study was performed in two phases. In both phases, the volunteers were asked to consume a "white meal", which is a meal without foods rich in BC (vegetables, fruits, cocoa, and plant-based drinks) the day before the experiment (Table S1). Seventy-two hours before the experiment, volunteers were asked not to consume alcohol or perform any kind of intense physical activity such as cycling and running. Study phase 1 aimed to set a controlled environment in which all volunteers would be offered the same food menu at the same time. For this, the volunteers (n = 12) were hosted for 2 days in a hotel. At day 1, blood samples were collected at fasting (T0) and 3 h after (T3) the consumption of 250 mL of placebo drink (PB). Similarly, on day 2, blood samples were collected at fasting and 3 h after the consumption of 250 mL of PS juice. Blood sampling and further biochemical analyses were performed by the Sabin clinical analysis laboratory, Brasilia. The results of the data obtained in study phase 1 are reported in Sections 3.2 and 3.3.

After the first intervention, the study phase 2 aimed to investigate the effect of the consumption of PS juice on gene expression in the volunteers. The same volunteers were asked to consume the same "white meal" as in study phase 1 (Table S1). The volunteers were invited to participate in a randomized crossover trial in which they had to acutely consume the same two beverages (250 mL PB or PS) in an interval of a 10-day washout period for the nutrigenomic study. This phase was performed at the Laboratory of Cellular Immunology, Faculty of Medicine, University of Brasilia, Brasilia. The results of the data obtained in study phase 2 are reported in Sections 3.4–3.6. For each experimental period, the fasting volunteers consumed either 250 mL of placebo drink (PB) or of *P. setacea* (PS) at the moment of their arrival in the morning, and blood samples were collected 3 h later.

This study was performed with the approval of the National Health Research Ethics Committee (CONEP, Brasilia, Brazil), protocol number 36348114.3.0000.0030, and all the volunteers provided their written informed consent. Description of the study can be found on ensaiosclinicos.gov.br RBR-84z83n.

2.3. Blood Sampling and Treatment

From the blood sampled in study phase 1, serum and plasma fractions were prepared to quantify metabolic markers (including glucose, insulin, homeostasis model assessment of β-cell function

(HOMA-BETA), homeostatic model assessment for insulin resistance (HOMA-IR), total cholesterol, high-density lipoprotein (HDL), low-density lipoproteins (LDL) and total triglycerides) and cytokines. The collection of biological samples and the biochemical analysis were conducted by Sabin laboratory on the same day. Blood sampling was also collected in heparin tubes and stored at −80 °C for the later quantification of cytokines. In study phase 2 (Laboratory of Cellular Immunology), blood samples were collected in heparin tubes for further nutrigenomics analysis on isolated PBMC. A total of 8 mL of venous blood was collected from volunteers using BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA), and PBMCs were isolated. Briefly, the tubes were immediately centrifuged at room temperature for 20 min at 1500 × *g*. After centrifugation, the cell layer containing PBMCs was collected and washed twice with sterile phosphate-buffered saline (PBS) with centrifugation at 300× *g* for 10 min after each washing step. The cell pellet obtained was immediately frozen at −80 °C and kept at this temperature until use.

2.4. Biochemical Parameters and Cytokines Analysis

The biochemical analyzes were conducted by Sabin Laboratory, Brasilia. To evaluate glucose, the hexokinase method was used; as for insulin, the insulin chemiluminescent immunoassay was applied, then HOMA BETA and HOMA IR were calculated. Total cholesterol was verified by means of the Allain's method of esterase/oxidase [31], HDL by using the direct method, LDL through the Martin–Hopkins's calculation, and total triglycerides by means of the oxidase/peroxidase method.

To quantify the circulating cytokines, serum samples were used to measure interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor (TNF), interferon-γ (INF-γ), and interleukin-17 (IL-17) protein levels by using the CBA Human T-cell TH1/TH2/TH17 Cytokine kit (Becton Dickinson, Franklin Lakes, NJ, USA). This method used bead array technology to simultaneously detect multiple cytokine proteins in the samples by flow cytometry. All the analyses were executed according to the manufacturer's guidelines. Shortly, cytokine capture beads were mixed with the plasma samples and incubated with phycoerythrin (PE)-conjugated detection antibodies to form sandwich complexes. The FCAP Array software was used to generate results in graphical and tubular format.

2.5. Total RNA Extraction

Total RNA extraction from PBMC was performed by using RNeasy Mini Kit, as recommended by the manufacturer (Qiagen, Hilden, Germany). The RNA quality was checked by means of 1% agarose gel electrophoresis, whereas the quantity was checked through absorbencies at 260 and 280 nm on NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA samples were stored at −80 °C until use.

2.6. Microarray Analyses and Bioinformatic Analysis

Total RNA (50 ng per sample) was amplified and fluorescently labeled to produce Cy5 or Cy3 complementary RNA (cRNA) by using the Low Input Quick Amp Labeling Two-Color Kit (Agilent, Santa Clara, CA, USA) in the presence of a two-color spike-in control, as recommended by the manufacturer. After purification, 825 ng of labeled cRNA was hybridized onto G4845A Human GE 4x44K v2 microarray (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. The G4845A Human GE 4x44K v2 microarray contains 27,958 Entrez Gene RNA sequences. After hybridization, an Agilent G2505 scanner (Agilent, Santa Clara, CA, USA) was used to scan microarrays. The hybridization data were extracted by means of the Feature Extraction software version 11.0 and analyzed through the GeneSpring GX software version 14.5 (Agilent Technologies, Santa Clara, CA, USA). Data were normalized using 50th percentile shift and analyzed with moderated *t*-tests corrected by Westfall–Young permutation with corrected *p*-value cut-off set to 0.05. All transcripts presenting $p < 0.05$ were considered differently expressed.

2.7. Bioinformatic Analyzes

For biological interpretation of the differentially expressed genes, we first performed Gene Ontology (GO) analyses using DAVID (Database for Annotation, Visualization and Integrated Discovery v6.7). The GO results were grouped on the basis of their functionality by using the online REVIGO software. The partial least squares discriminant analysis (PLSDA) plot was obtained through MetaboAnalyst (https://www.metaboanalyst.ca). Gene networks were built with a data-mining approach using the Metacore software, and gene pathway analyses of the Kyoto Encyclopedia of Genes and Genomes (KEGG) and BioCarta databases were conducted by using the Genetrial2 online tool.

2.8. Statistical Analyses

The data obtained were previously analyzed for normality through D'Agostino's and Pearson's tests. The outlier values were calculated by means of the Tukey test and excluded from the analyses only when interfering with normality values. For two independent groups, paired Student's *t*-test was applied to samples that had a normal distribution, and for those without normal distribution, Wilcoxon's *t*-student test was applied. Descriptive values were expressed as mean ±SD corrected. The differences between the variables compared were considered statistically significant when the bi-tailed probability of their occurrence due to chance (type I error) was less than 5% ($p < 0.05$). Analyses and graphs were performed by using the GraphPad Prism 7 software for Mac (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Volunteers' Baseline Characteristics

The baseline characteristics of the volunteers are summarized in Table 1. The subjects enrolled were men with a mean age of 48.66 ± 6.82 years that were overweight or slightly obese (BMI ranging from 25.00 to 30.80) with a mean waist circumference of 96.83 ± 6.49 cm. The subjects ranged from normal to slightly hyperglycemic ($n = 1$, 103 mg/dL), as well as from normal to mildly hyperlipidemic ($n = 2$), as shown by the values for plasma total triglycerides (Table 1). All the other parameters were within normal range. Two of the 12 volunteers presented three to four factors that may define them as having a metabolic syndrome (waist circumference ≥ 90cm; serum triglycerides ≥ 150 mg/dL mmol/l; HDL cholesterol < 40 mg/dL; and fasting plasma glucose (FPG) ≥ 100 mg/dL). Statistical tests without these volunteers were therefore remade and the statistical significances did not change.

Table 1. Volunteers' baseline characteristics ($n = 12$).

Parameter	Mean ± SD (Range)
Age, years	48.66 ± 6.82 (41–62)
BMI, kg/m^2	28.18 ± 2.08 (25.0–30.8)
Waist circumference, cm	96.83 ± 6.49 (88–112)
Fasting glucose, mg/dL	90.83 ± 5.54 (83-103)
Basal insulin, µUI/mL	11.14 ± 3.58 (5.5–17.7)
HOMA IR	2.49 ± 0.77 (1.2–3.7)
HOMA BETA	152.8 ± 71.58 (73–304)
Triglycerides, mg/dL	116.58 ± 57.55 (49–218)
Total cholesterol, mg/dL	188.17 ± 33.40 (115–232)
HDL cholesterol, mg/dL	47.91 ± 10.67 (38–72)
LDL cholesterol, mg/dL	117.00 ± 27.09 (60–156)
Apoliprotein A, mg/dL	133.67 ± 20.95 (107–172)
Apoliprotein B, mg/dL	101.77 ± 24.80 (49–133)
Creatinin, mg/dL	0.97 ± 0.14 (0.7–1.2)
TGO, U/L	20.67 ± 2.87 (14–25)
TGP, U/L	25.41 ± 6.97 (11–35)

3.2. Effect of Passiflora setacea Juice on Glucose and Lipid Metabolism (Phase 1)

Glucose, insulin, HOMA IR, triglycerides and HDL in plasma were analyzed before (T0) and 3 h after (T3) the intake of placebo and PS juice. The data show that insulin and HOMA IR levels decreased

statistically 3 h after PS juice intake ($p = 0.0068$ and $p = 0.001$, respectively), whereas no significant change was observed after the placebo intake (Figure 1A). The plasma glucose concentrations decreased in a similar way after the intake of the two drinks. The high-density lipoprotein (HDL) level increased significantly after PS juice consumption ($p = 0.0280$), whereas no change was observed after PB drink ($p = 0.3541$), as seen in Figure 1B. No effects of PS or PB were detected on total and LDL cholesterol levels.

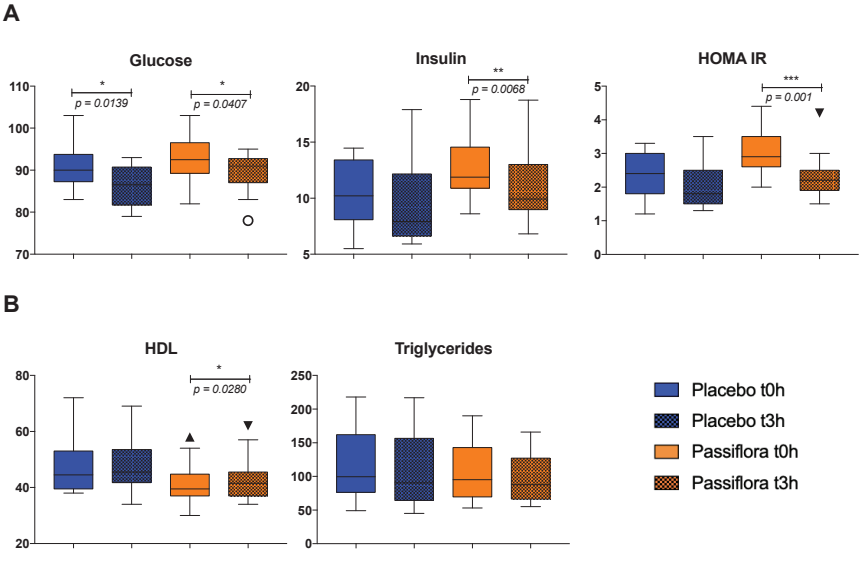

Figure 1. Acute effect of *Passiflora setacea* juice and placebo drink consumption on glucose (**A**) and lipid (**B**) metabolism markers in overweight volunteers ($n = 12$). Results analyzed by means of the non-parametric paired *t*-test (Wilcoxon's test), medians, and SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3. Effect of Passiflora setacea Juice Intake on Cytokine Serum Levels (Phase 1)

We determined the effect of PS juice intake on cytokine serum levels. Data showed that the IL-17A level did not increase after 3 h of PS juice consumption ($p = 0.2962$); however, it increased after placebo consumption ($p = 0.0124$) (Figure 2). We also observed that TNF-α presented a similar but not significant pattern as IL-17A, that is, its level tended to increase after PB drink ($p = 0.0645$), whereas it remained unchanged after PS juice ($p = 0.5489$) (Figure 2). There were no statistical changes in the other cytokine measures of IL-2, IL-4, IL-6, IL-10, and INF-γ (Figure 2).

3.4. Passiflora setacea Modulated Gene Expression in Circulating Cells (Phase 2)

Following RNA extraction and quality control of both RNA and microarray hybridization, we obtained good quality RNA from 8 out of 12 volunteers. To access the nutrigenomic effect of an acute intake of PS juice in PBMCs, we performed a pangenomic gene expression analysis 3 h after PS juice and PB drink consumption for the eight volunteers. Comparison of global gene expression profiles obtained for the volunteers by using PLSDA showed the separation of profiles between the two groups, suggesting different gene expression profiles between the volunteers that consumed PS and the volunteers that consumed PB (Figure 3). This observation suggests differential modulation in expression of genes after an acute intake of PS juice compared to the control drink.

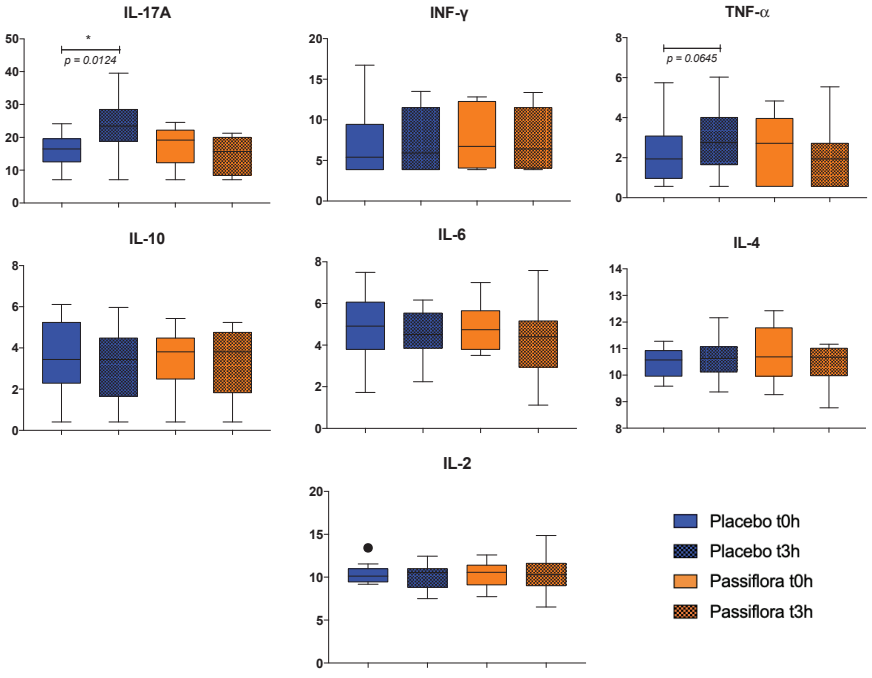

Figure 2. Acute effect of *Passiflora setacea* juice consumption on cytokine serum levels in overweight volunteers (n = 12). Results analyzed by means of the non-parametric paired t-test (Wilcoxon's test). * $p \leq 0.05$, • outlier tested by Tukey.

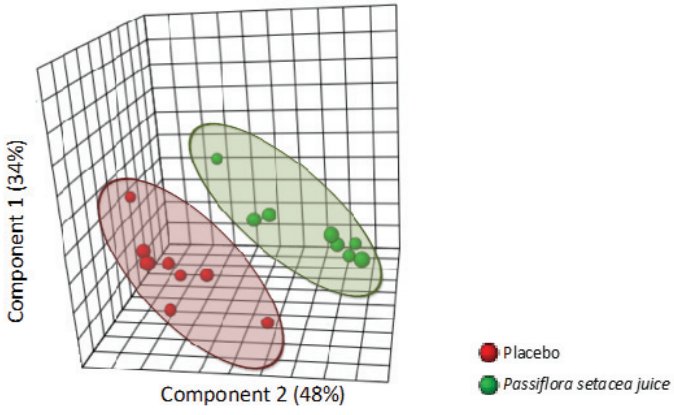

Figure 3. The comparison of the global gene expression profiles obtained for the volunteers using partial least squares discriminant analysis (PLSDA) shows the separation of profiles between the two groups, and suggests different gene expression profiles between the volunteers that consumed *Passiflora setacea* juice (PF) and the volunteers that consumed placebo (PB; placebo).

Following this observation, we performed a statistical analysis to identify which genes had their expression altered after the consumption of PS compared to PB. Statistical analyses identified 1327 genes presenting changes in their expression after PS consumption. Among them, most genes were identified as having their expression down-regulated with the average fold-change for up-regulated genes being

2.48 and for down-regulated genes being −2.15. Among the genes showing the highest differential modifications were *TMEM151A*, *MLPH*, *MYH2*, *SERPINA9*, or *FNA21*.

For the biological interpretation of the differentially expressed genes, we first performed Gene Ontology (GO) analyses using DAVID database, and the GO were then clustered into function groups by using the online REVIGO software. This showed that differentially expressed genes are involved in various biological processes such as calcium ion transmembrane transport (potassium ion transport and phospholipid efflux), cell differentiation (extracelular matrix organization and histone lysine methylation), G-protein-coupled receptor signaling pathway (chemical synaptic transmission and neuropeptide signaling pathway), cell adhesion, and transcription from RNA polymerase promoter (Figure S1). This analysis revealed that the consumption of PS juice modulated the expression of genes presenting different biological functions.

To deepen the identification of the functions and cellular processes potentially affected by the consumption of PS juice, we performed gene network analyses of the differentially expressed genes. Gene networks, built through data-mining using the Metacore software, suggested, as did the GO analyses, that the consumption of PS juice changed the expression of genes involved in cellular function. Among the most over-represented networks were those involved in calcium and potassium transport, cell adhesion and cell–matrix interactions, neurogenesis, or transmission of nerve impulse (Figure 4). The genes identified in these networks were *NMDA* receptor, *matrix metalloproteinase (MMP)-7*, *ADAMTS9*, *mGluR*, *CaMKII* alpha, *CACNA1C*, and *SLC24A2*. These genes decode proteins involved in vascular tissue damage, in the reduction of insulin sensitivity and secretion, and in neuropsychiatric disorders and neuron excitability.

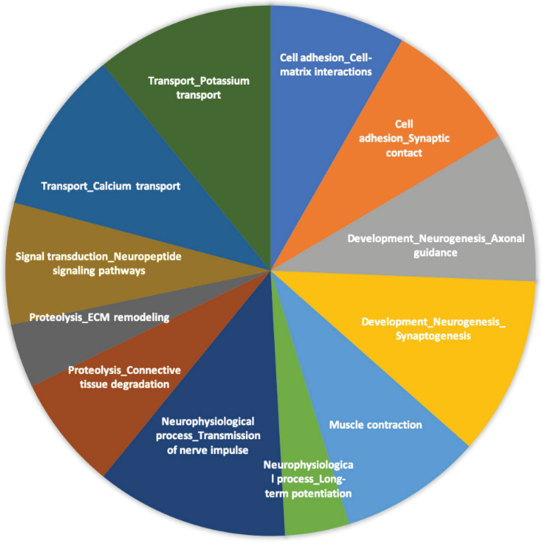

Figure 4. Networks enriched with differentially expressed genes in volunteers' peripheral blood mononuclear cells in response to *Passiflora setacea* pulp consumption. Gene networks were identified using MecaCore software that uses text mining approach to build gene–gene interactions and identify their cellular functions.

Besides the network analyses, we also performed gene pathway analyses employing the use of the Genetrial2 online tool by searching the KEGG and BioCarta databases. As shown in Figure 5, the differentially expressed genes identified are involved in cellular pathways including inflammation, metabolism, cell signaling, and neurofunction-related processes. Regarding the pathway related to inflammation, we identified a cytokine–cytokine receptor, cell adhesion molecules, and chemokine signaling pathways,

which include genes such as *TNFSF18*, *IL36A*, *JAM2*, *ADCY8*, or *CCL16*. In pathways related to cellular metabolism, we identified circadian entrainment, insulin secretion, and *P13K-Akt* signaling pathways, which include genes such as *GRIN2A*, *PRKG1*, *CACNA1D*, *GLP1R*, *G6PC2*, and *LAMA1*. Calcium and adrenergic signaling pathways were also identified, containing *PTGER3*, *ADCY8*, and *CACNA1D* genes. Several pathways related to neurofunction were also identified such as glutamatergic synapse, neuroactive ligand-receptor interaction, and *MAPK* signaling pathways, in which genes such as *GABRG1*; *glutamate receptor*, *ionotropic*, *NMDA1* (*GRIN1*); *CACNA1D*; and *ADCY8* were mapped.

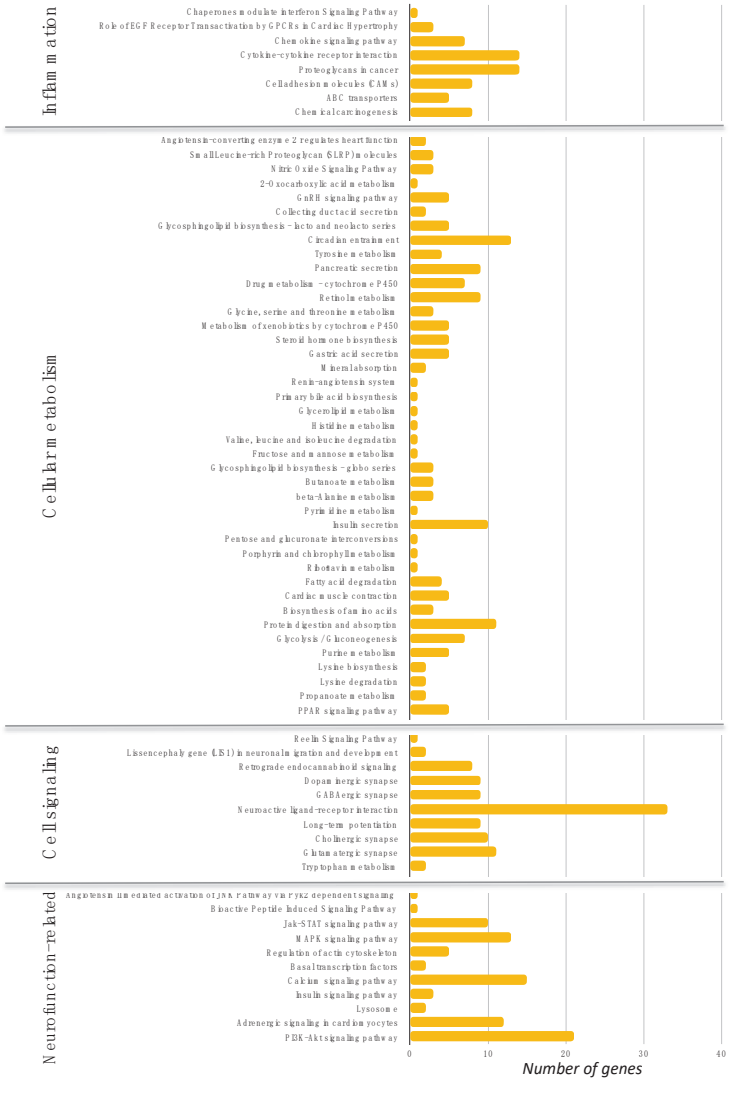

Figure 5. Significantly enriched pathways with differentially expressed genes in volunteers' PBMC in response to PS pulp consumption. Pathways were identified using Genetrial2 online tool and KEGG database, and were grouped regarding their functions. *x*-axis represents the number of genes in each pathway.

3.5. Protein–Protein Interaction (Phase 2)

Apart from the bioinformatics analyses on cellular networks and pathways of differentially expressed genes identified, we also searched for protein–protein interactions. We observed interactions among the genes whose expression were affected by the consumption of PS. By using the online String database, we identified 1013 nodes and 3066 edges with 6.05 nodes on average (Figure S2). Among them, 39 genes showed over 15 interactions with other proteins, making them important nodes in the protein–protein interactome. These 39 genes revealed over 700 interactions, which are a fifth of the total number verified. This suggests that changes in the expression of these genes can have an important impact on protein interactome and consequently on cell function. These genes are involved in the cellular pathways regulating processes such as cyclic adenosine monophosphate (cAMP) signaling pathway, nitric oxide signaling pathway, dopaminergic synapse, insulin, or PI3K-Akt signaling pathways. Proteins interacting with these 32 genes have been searched and 256 genes have been identified. Pathway analyses of these genes showed that they are involved in pathways such as the cAMP signaling pathway, PI3K-Akt signaling pathway, Ras/Rap1 (Ras-related protein 1) signaling, insulin secretion, cytokine–cytokine receptor interaction, chemokine signaling pathway, retrograde endocannabinoid signaling, or regulation of actin cytoskeleton.

3.6. Transcriptional and Post-Transcriptional Regulators of the Nutrigenomic Effect (Phase 2)

The bioinformatics analyses of gene expression data were further performed with the aim to identify potential transcription factors involved in the mediation of the PS juice nutrigenomic effect observed. The most significant transcription factors (Figure 6) were cAMP Responsive Element Binding Protein 1 (CREB1), nuclear factor-kappa B (Nf-kB), and specificity protein 1 (SP1). These transcription factors are involved in gluconeogenesis regulation, lipid metabolism, and insulin signaling pathways. They are also associated with vascular calcification, pathogenesis of type 2 diabetes (TD2), and diabetic cardiovascular disease. Other transcription factors are Proto-Oncogene C-Jun (c-jun), Signal Transducer And Activator of Transcription 3 (STAT3), and tumor protein P53 (p53).

	Network	*p*-Value
1	CREB1	4,21E-289
2	RelA (p65 NF-kB subunit)	6,71E-286
3	SP1	3,25E-279
4	ESR1 (nuclear)	3,37E-219
5	c-Jun	4,07E-208
6	Androgen receptor	6,69E-206
7	Oct-3/4	1,8E-201
8	NRSF	2,94E-199
9	LHX2	1,28E-192
10	p53	1,28E-192
11	GATA-3	1,28E-192
12	C/EBPbeta	3,42E-188
13	STAT3	3,42E-188
14	c-Myc	1,76E-183
15	HNF4-alpha	7,8E-177
16	GCR	6,28E-175
17	HIF1A	1,02E-172
18	C/EBPalpha	4,29E-166
19	DBP	6,92E-164
20	HNF1-alpha	1,8E-159
21	SP3	4,64E-155
22	HNF3-beta	1,19E-150
23	SRF	1,91E-148
24	EGR1	3,06E-146
25	c-Fos	4,9E-144
26	p63	1,25E-139
27	NANOG	1,25E-139
28	GATA-4	1,25E-139
29	AHR	1,99E-137
30	TCF7L2 (TCF4)	1,99E-137

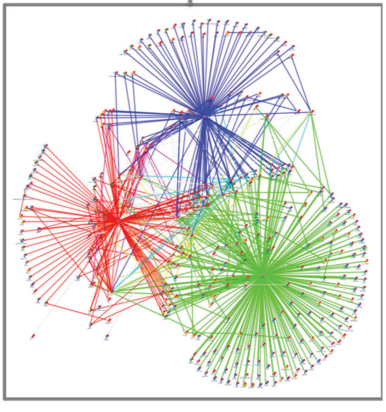

Figure 6. Bioinformatics analyses of potential transcription factors involved in the mediation of the PS juice's nutrigenomic effect observed. Transcription factors were identified using MetaCore algorithm and the most significant transcription factors listed were CAMP Responsive Element Binding Protein 1 (CREB1), nuclear factor-kappa B (Nf-kB), and specificity protein 1 (SP1). On the right, the interactions of the networks with these three transcription factors identified are represented.

Besides the transcriptional regulators potentially involved in the nutrigenomic effect observed, we also searched for potential post-transcriptional regulators of the gene expression, particularly microRNA. Using the online OmicsNet tool, we identified over 30 microRNAs (miRNAs) that could interact with differentially expressed genes and regulate their expression at the post-transcriptional level (Table 2). This suggests that PS juice consumption could potentially regulate the expression of microRNAs and consequently affect levels of mRNA of genes we identified as differentially expressed. Using the same online tool, we then performed integrated analyses of the identified differentially expressed genes, potential transcription factors, and potential microRNAs (Figure 7). We observed a network of interaction among these three levels of regulation of cell function, suggesting that PS juice consumption can significantly impact immune cells at the molecular level and consequently impact their functions.

Table 2. microRNA list.

hsa-mir-335-5p	MIMAT0000765	228
hsa-mir-26b-5p	MIMAT0000083	193
hsa-mir-16-5p	MIMAT0000069	161
hsa-mir-92a-3p	MIMAT0000092	161
hsa-mir-124-3p	MIMAT0000422	152
hsa-mir-155-5p	MIMAT0000093	116
hsa-mir-93-5p	MIMAT0000646	116
hsa-mir-1-3p	MIMAT0000416	114
hsa-mir-17-5p	MIMAT0000070	113
hsa-mir-615-3p	MIMAT0003283	112
hsa-let-7b-5p	MIMAT0000063	104
hsa-mir-106b-5p	MIMAT0000680	101
hsa-mir-20a-5p	MIMAT0000075	99
hsa-mir-218-5p	MIMAT0000275	99
hsa-mir-1-1	MI0000651	98
hsa-mir-484	MIMAT0002174	90
hsa-mir-193b-3p	MIMAT0002819	89
hsa-mir-15b-5p	MIMAT0000417	88
hsa-mir-15a-5p	MIMAT0000068	81
hsa-mir-20b-5p	MIMAT0000076	80
hsa-mir-21-5p	MIMAT0001413	80
hsa-mir-30a-5p	MIMAT0000087	79
hsa-mir-186-5p	MIMAT0000456	78
hsa-mir-519d-3p	MIMAT0002853	77
hsa-mir-24-3p	MIMAT0000080	76
hsa-mir-320a	MIMAT0000510	75
hsa-mir-8485	MIMAT0033692	75
hsa-mir-192-5p	MIMAT0000222	74
hsa-mir-195-5p	MIMAT0000461	73

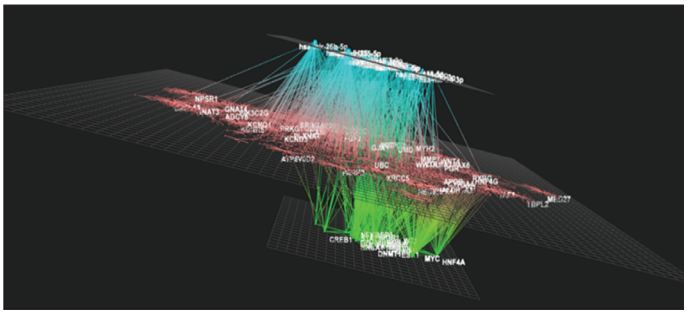

Figure 7. Bioinformatics analyses of potential miRNA involved in the mediation of the *Passiflora setacea* juice's nutrigenomic effect observed. OmicsNet online tool was used to identify interactions between the differentially expressed genes identified (in pink) with the potential transcription factors identified (in green), and potential miRNAs involved (in blue).

4. Discussion

This is the first time that the effect of a Brazilian savannah fruit was described on IL-17A blood levels and on gene expression profile in humans. We found that the consumption of one serving of PS juice (similar composition of a whole fruit but without its seeds) statistically decreased the levels of insulin and HOMA IR while increasing HDL levels.

It is known that disorders in insulin metabolism and consequently in glucose metabolism result in oxidative stress and inflammation, which lead to micro- and macrovascular dysfunctions and to the further development of diabetes and cardiovascular diseases [5]. These complications are associated with endothelial dysfunction, pro-inflammatory cytokines, reactive oxygen species formation, and adhesion molecule production [32]. This process results in the increase in the adhesion of immune cells to endothelial cells, as well as in their transendothelial migration into the vascular wall, which are the initial steps to the development of atherosclerosis, the origin of all vascular-related diseases. Therefore, by changing the insulin, HOMA IR, and HDL levels, PS juice could exert anti-inflammatory and vasculo-protective properties and consequently prevent or delay the onset of associated diseases. This observation could be related to the results of other in vivo and in vitro studies that have shown the potential of dietary polyphenols on insulin response. For example, isoorientin, a flavonoid found in PS, has proven to revert insulin resistance in adipocytes by stimulating the proper phosphorylation of proteins in the insulin signaling pathway [33]. Another path of action may be explained by the inhibition of the key enzymes involved in starch digestion (alpha-amylase and alpha-glucosidase) by polyphenols [34]. The caffeic acid, a phenolic acid also present in PS has shown the capacity to inhibit these enzymes [35]. Polyphenols from water chestnut husk [36], green tea [37], and apple [38] have been reported to reduce serum insulin levels in normal mice. However, few studies have also shown the effect of whole food on human insulin metabolism. Nyambe-Silavwe and Williamson [39] reported the effect of dried fruits with green tea in the decrease of insulin serum levels in healthy volunteers.

We also observed an increase of blood HDL concentrations after PS juice intake that was not detected within the PB condition. Recent nutrition intervention studies on polyphenol-rich foods such as olive oil [40] or dark chocolate [41] have been shown to positively affect HDL levels in humans. One possible explanation is that these polyphenol-rich foods may induce changes in the biochemical properties of the lipoprotein that contribute to its main biological function, particularly in the enhancement of the cholesterol efflux capacity [42]. It can therefore be suggested that PS consumption modulates risk factors of cardiometabolic diseases.

This study also revealed that an acute intake of PS juice kept IL-17A at a basal level and indicated a tendency to decrease TNF-alpha levels ($p = 0.0645$) when compared to PB condition. The IL-17A is a pro-inflammatory cytokine that stimulates neutrophil inflammatory response [43] and the production of other pro-inflammatory cytokines such as TNF-alpha, IL-1B, and IL-6 [44], as well as the expression of adhesion molecules such as Intercellular Adhesion Molecule 1 (ICAM-1) [45]. Its activities are vastly increased due to synergy with TNF-alpha that promotes the induction of target genes involved in inflammatory processes [46]. Cyanidin, a key flavonoid present in red berries, has shown the capacity to reduce inflammation in mice through binding with the extracellular domain of IL-17RA and consequently disrupting the IL-17A/IL-17RA complex formation [47]. Few studies have provided evidence regarding the role of diet in modulating IL-17 levels in humans. Peluso et al. [6] observed a drop of this cytokine in the plasma of 14 overweight subjects after a pineapple, blackcurrant, and plum juice consumption. Taken together, this observation suggests that PS consumption could present anti-inflammatory effects during the post-prandial period.

In the present study, using microarray analysis, we showed that the consumption of one single cup of PS juice by volunteers significantly affected PBMC gene expression profiles. Our study is the first to show the effect of a *Passiflora* species on the modulation of gene expression in humans. Another study has shown the capacity of another species of *Passiflora* in modulating gene expression in mice. Toda et al. [48] demonstrated that the aerial parts of the *Passiflora incarnata* Linnaeus extract

can modulate the expression of genes that may be involved in the prevention of obesity [49] and hyperglycemia [50]. Regarding the effects of BC found in PS on gene expression, it has been reported that isoorientin stimulated the transcription of genes encoding components of insulin signaling pathway in murine insulin-sensitive and insulin-resistant adipocytes [33]. Orientin from *Commelina communis* L. down-regulated the expression of peroxisome proliferator activated receptor (PPAR) and mRNA levels of genes involved in adipogenesis, lipogenesis, and triglyceride sysnthesis in vitro [51]. A plant extract rich in orientin, isoorientin, vitexin, and isovitexin has been shown to inhibit the mRNA levels of TNF-α also in vitro [52]. Therefore, our original study suggests that potential health benefits of PS could be related to its capacity to modulate the expression of genes in vivo in humans.

Bioinformatic analysis also revealed that PS consumption modulated the expression of a group of genes, 25, involved in the regulation of inflammation and immune response, particularly chemokine signaling pathway and cytokine–cytokine receptor interactions. Among these genes are CXCL17, IL36A, CCL16, CCL21, and IL-25. CCL16 is a pro-inflammatory chemokine that may be involved in the development of diseases such as irritable bowel syndrome [53]. Chemokines, a group of cytokines that attract and activate leucocytes into inflamed tissue, have been associated with the pathogenesis of a number of diseases, ranging from atherosclerosis to human immunodeficiency virus (HIV) infection [54], and CCL21 has been suggested as being involved in the pathogenesis of various inflammatory disorders including rheumatoid arthritis, inflammatory bowel diseases, and atherosclerosis [55]. Nutrigenomic analysis also identified several interleukins such as IL-36A that have pro-inflammatory properties and have been described as being involved in pulmonary inflammatory responses [56]. Several studies have shown that foods rich in BC or isolated BC such as hesperidin can regulate the expression of chemokines [7]. Taken together, the results suggest that the acute consumption of PS can present anti-inflammatory effects by modulating the expression of related genes.

This nutrigenomic study identified changes in the expression of genes involved in processes such as cell adhesion and cell–matrix interactions; chemokine signaling; insulin secretion; calcium and potassium transport; as well as inflammation, atherosclerosis development, and neurostimulation. Among them are genes encoding matrix metalloproteinases (MMPs), desintegrins, and metalloproteases (ADAMs), for which expression was identified as down-regulated. MMPs constitute a family of extracellular processing enzymes responsible for inflammation and acquired immunity [57]. Expression of genes encoding MMPs is frequently increased by cytokines, and reactive oxygen species are often involved in this mechanism [58]. MMP-7 over-expression regulates chemokine gradients that can lead to severe tissue damage through transepithelial influx of neutrophils [59]. In an in vitro and in vivo study, resveratrol reversed the injury of human epithelial cells and attenuated such injury in mice through the inhibition of MMP-7 expression [60]. Kinase Insert Domain Receptor (KDR) (or Vascular Endothelial Growth Factor Receptor-2, VEGFR2) is a key receptor that promotes Vascular Endothelial Growth Factor (VEGF) to form mitosis and generate vascularization. VEGF promotes proliferation and migration of cells and activates matrix matalloproteinase secretion [61], which can lead to exacerbation of tissue damage during inflammation. VEGF is highly expressed in tissues undergoing growth or remodeling in cancer and atherosclerosis [62]. KDR's expression was down-regulated with PS juice consumption, as well as JAM-2's (junctional adhesion molecule 2) gene involved in leukocyte recruitment and extravasation under inflammatory conditions [52]. Few studies have suggested the capacity of foods or BC to modulate the expression of these genes. A formulation of Chinese herbs was capable of downregulating the expression of KDR and VEGF in a mouse with hepatocellular carcinoma [63]. Monfoulet et al. [64] revealed the capacity of curcumin to reduce endothelial junctional permeability. Thus, the capacity of PS to down-regulate the expression of this gene suggests a potential lower interaction of immune cells with vascular endothelial cells, which represent the initial steps of atherosclerosis development. As atherosclerosis is associated with the genesis of others cardiovascular diseases, PS juice consumption may reveal interesting mechanisms underlying its potential vasculo-protective properties.

ADAMs constitute a family of proteases with cell adhesive potential [65] and other functions, including extracellular matrix (ECM) degradation, shedding of various cell surface proteins, and influence on cell signaling patterns [66]. ADAM12 is an active protease in ECM that causes changes in proliferation and differentiation of adipocyte maturation and also in the development of obesity induced by high-fat diet [67]. It has been shown to affect the insulin-like growth factor (IGF)/mTOR (mammalian target of rapamycin) and peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathways, leading to increased lipid accumulation in mature adipocytes [68]. ADAMTS9 is a risk gene for type 2 diabetes development and its over-expression is associated with impaired insulin signaling in peripheral tissues and also with insulin resistance [69]. Its risk allele (rs4607103 C) has been demonstrated to decrease mitochondrial function and to alter glucose and lipid metabolism [70]. Another gene identified as differentially expressed after PS juice consumption is adenylate cyclase 8 (ADCY8), which is involved in insulin secretion and glucose homeostasis. Sung et al. [71], in a genome-wide association analysis, associated the ADCY8 gene with obesity and abdominal visceral fat depot. Considering all these previous factors, the gene expression profile obtained after an acute consumption of PS suggests a lower accumulation of lipids in PBMCs and a lower impairment in insulin signaling, presenting potential molecular targets of PS juice consumption and their potential health properties.

Besides the modulation of genes related to cardiometabolic regulations, our nutrigenomic analysis also identified the fact that several genes modulated by the acute consumption of PS were associated with neurofunction, such as CACNA1C, GRIN1, and G protein-coupled receptor 50 (GPR50). The immune-to-brain and brain-to-immune communication has been recently studied [72]. The central nervous system can communicate with peripheral monocytes, promoting gene expression modulation, particularly with regards to the NF-kB transcriptional control pathway [73]. The CACNA1C is a gene contributes to the etiology of psychiatric disorders and to phenotypes affected by those conditions such as memory and circadian rhythms [74]. Such symptoms are present in a proportion of the general population and are correlated with poorer cognitive performance and with adverse health outcomes [75] such as atherosclerosis [76]. The GRIN1 (glutamate receptor, ionotropic, NMDA1) gene plays an important role in excitatory neurotransmission, and the increase of its expression has been associated with anxiety in response to stress in mice [77]. Another gene whose expression has been modulated after PS juice consumption was GPR50, a gene involved in late-life depression in certain subgroups of depressed individuals [78]; its down-regulation is associated with torpor enhancement [79]. Acute consumption of PS juice decreased the expression of these genes, which suggests that PS consumption may affect torpor, a hypothesis that can explain PS's popular name "sleep passionfruit". These observations suggest the potential effect of PS consumption as an auxiliary treatment for cognitive functions and for psychiatric disorders.

The three major transcription factors whose activities might be affected by PS consumption and that are possibly involved in the nutrigenomic effect observed are CREB1, RELA Proto-Oncogene (RelA), and SP1. CREB1 regulates gluconeogenesis, lipid metabolism, and insulin signaling pathways, and the activity of its transcriptional promoter is associated with the pathogenesis of TD2 [80], adipogenesis [81], and major depressive disorder [82]. RelA is a sub-unit of NF-κB, a transcription factor critical for the expression of proinflammatory cytokines in human monocytes. Studies on the effect of bioactive compounds (BC) on this transcription factor showed that orientin, isoorientin, vitexin, and isovitexin exert suppressive action of these compounds upon NF-κB activation [24]. Elevated SP1 plays a pro-apoptotic effect and stimulates vascular calcification [83]. It has been observed that the BC (-)-epigallocatechin-3-gallate can modulate the activity of this transcription factor [84]. Therefore, the capacity of PS juice consumption to affect the activity of these transcription factors could present major regulatory mechanisms observed in nutrigenomic modifications underlying their health properties.

Literature lacks data about the effect of *Passiflora* on miRNA expression. However, it has been suggested that plant food bioactives can modulate the expression of miRNA [85,86]. Among the

miRNA identified from bioinformatic analysis as potentially involved in the post-transcriptional regulation of identified differentially expressed genes by PS juice consumption, we can list miRNA-16, -26, and -124. miRNA-16 has shown to play a role in inflammation [87] and hypertension [88], whereas miRNA-124 mediates anti-inflammatory effects and is involved in neuroprotective mechanisms [89]. Therefore, by potentially modulating the expression of these miRNAs, PS consumption can regulate genes involved in the development or the prevention of cardiometabolic and neurological diseases.

5. Conclusions

This acute study has provided the first clinical data supporting an interest in PS pulp consumption for human health. The positive changes we observed in some inflammatory and metabolic biomarkers as well as in the PBMC gene expression profile after an acute intake of one serving of PS pulp by overweight, middle-age volunteers suggest potential anti-inflammatory and anti-diabetic effects. These results, originating from an acute study, cannot be directly extrapolated to chronic consumption of PS pulp. However, it opens interesting perspectives for future long-term clinical studies using PS products in order to better characterize their health properties and identify the compounds/components responsible for these biological effects. Furthermore, developing this research line is important to increase knowledge on the role of plant foods derived from Brazilian biodiversity in the prevention or delay of the onset of chronic diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/4/1104/s1: List S1: Placebo ingredients. Table S1: "White meal" menu.

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nutrients

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Dietary Influence on Systolic and Diastolic Blood Pressure in the TwinsUK Cohort

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Abstract: Nutrition plays a key role in blood pressure (BP) regulation. Here, we examine associations between nutrient intakes and BP in a large predominantly female population-based cohort. We assessed the correlation between 45 nutrients (from food frequency questionnaires) and systolic BP/diastolic BP (SBP/DBP) in 3889 individuals from TwinsUK not on hypertensive treatments and replicated in an independent subset of monozygotic twins discordant for nutrient intake (17–242 pairs). Results from both analyses were meta-analysed. For significant nutrients, we calculated heritability using structural equation modelling. We identified and replicated 15 nutrients associated with SBP, 9 also being associated with DBP, adjusting for covariates and multiple testing. 14 of those had a heritable component (h^2: 27.1–57.6%). Strong associations with SBP were observed for riboflavin (Beta(SE) = −1.49(0.38), $P = 1.00 \times 10^{-4}$) and tryptophan (−0.31(0.01), $P = 5 \times 10^{-4}$), while with DBP for alcohol (0.05(0.07), $P = 1.00 \times 10^{-4}$) and lactose (−0.05(0.0), $P = 1.3 \times 10^{-3}$). Two multivariable nutrient scores, combining independently SBP/DBP-associated nutrients, explained 22% of the variance in SBP and 13.6% of the variance in DBP. Moreover, bivariate heritability analysis suggested that nutrients and BP share some genetic influences. We confirm current understanding and extend the panel of dietary nutrients implicated in BP regulation underscoring the value of nutrient focused dietary research in preventing and managing hypertension.

Keywords: nutrients; hypertension; blood pressure; management; prevention; diet

1. Introduction

Hypertension is the most prevalent modifiable risk factor for cardiovascular (CVD) morbidity and mortality [1]: for every 10 mmHg reduction in systolic blood pressure (SBP), the risk decreases by 11% [2,3]. Yet, hypertension prevalence is mounting, with a 1.5 fold increase estimated by the year 2025, affecting 1.5 billion individuals [4]. Blood pressure (BP) is determined by complex interactions between genetics and environmental exposures [5]. Some environmental factors are non-modifiable, such as age, ethnicity and gender [6]. However, others are modifiable, with extensive evidence supporting lifestyle modification, including physical activity, smoking and particularly dietary changes as efficacious first-line therapies for hypertension [7]. Studies have reported that adhering to a Med/DASH diet improves CVD outcomes [8], and recently there has been a push to move research towards whole dietary patterns [9]. However, due to the complexity of dietary patterns, many important nutrient effects may be overlooked.

Numerous studies have evidenced the relative effects of single nutrients on BP, including salt, potassium and alcohol. For instance, Cochrane and collaborators found that a 4.4 g/day reduction in salt (1733 mg sodium) reduced BP by 4.18/2.06 mmHg [10], which was significantly higher in hypertensive individuals (5.39/2.82 mmHg) compared to normotensives (2.42/1 mmHg) [10]. Additionally, a meta-analysis of 29 randomised clinical trials (RCTs) showed that an increase in potassium of ≥20 mg/d led to a BP reduction of 4.9/2.7 mmHg, including trials with hypertensive and normotensive subjects [11]. These results highlight the value of nutrient based research to control BP.

In the present study, we assess the role of 45 nutrient intakes estimated from Food Frequency questionnaires on SBP and diastolic BP (DBP) in a large cohort of twins. Having identified nutrient intakes associated with SBP or DBP, we validated the results using identical twins discordant for that particular nutrient. This allowed us to isolate the non-genetic contribution of nutrient intakes upon blood pressure. Finally, given the twin nature of our data, we estimated heritability of the associated nutrient intakes.

2. Methods

2.1. Study Population

Study participants were twins enrolled in the TwinsUK registry, a national register of adult twins recruited as volunteers without selecting for particular disease or traits [12]. We included 3889 predominantly female twins that completed a 131-item validated food frequency questionnaire (FFQ) between 1996 and 2015 [13] and had a concurrent BP measurement (within 0.16(SD = 0.29) years).

Twins provided informed written consent and the study was approved by St. Thomas' Hospital Research Ethics Committee (REC Ref: EC04/015). Data relevant to the present study include SBP/DBP, BMI and zygosity (determined by methods previously outlined [14]).

2.1.1. Assessment of Blood Pressure

Clinic BP was measured by a trained nurse using either the Marshall mb02, the Omron Mx3 or the Omron HEM713C Digital Blood Pressure Monitor performed with the patient in the sitting position for at least 3 min. At each visit, the cuff was placed on the subject's arm so that it was approximately 2–3 cm above the elbow joint of the inner arm, with the air tube lying over the brachial artery. The subject's arm was placed on the table or supported with the palm facing upwards, so that the tab of the cuff was placed at the same level of the heart. Triplicate measurements were taken with an interval of approximately 1 min between each reading, with mean of second and third measurements recorded.

2.1.2. Nutrient Data

Intakes of 46 nutrients were estimated from a validated 131-item food frequency questionnaire (FFQ) based upon the EPIC FFQ [13]. Prior to analysis intake frequencies were adjusted for total energy intake using the residual method [15]. FFQs were then coded and processed using FETA [16], an open-source, cross-platform tool designed to process dietary data from the EPIC FFQ, in accordance with their guidelines. The default nutritional database of which is based on the McCance and Widdowson's The Composition of Foods (5th edition) [17].

2.1.3. Dietary Indices

To determine the effects of the whole diet, we employed the most prominently used dietary indices including, the NOVA classification system [18], the Healthy Eating Index (HEI) [19], the alternate Healthy Eating Index (aHEI) [20], the Dietary Approaches to Stop Hypertension (DASH) score [21], the Alternate Mediterranean Diet Score (aMED) [22], the Dietary Quality Index International (DQI-I) [23], the Plant Diversity Index (PDI) [24], the Healthy PDI (hPDI) [24] and the Unhealthy PDI (uPD) [24]. Descriptions of the indices can be found in Table S1.

2.2. Statistical Analysis

Statistical analysis was performed using R version 3.6.2.

Linear mixed models were used to investigate the associations of each nutrient with SBP and DBP in the discovery sample (excluding monozygotic (MZ) twins with nutrient intake over one SD apart). Analyses were adjusted for age, gender, BMI, family relatedness and multiple testing using Benjamini–Hochberg correction (FDR < 0.05).

The MZ discordant twin pairs were then used to replicate the significant findings from the discovery group. Associations that passed the 5% level of significance or were in the same direction as the discovery group were considered replicated. Finally, we combined the results of both analyses using an inverse variance fixed effect meta-analysis.

A backwards stepwise regression, including the BP-associated nutrients, was then employed in the overall sample to identify nutrient intakes independently associated with SBP and DBP ($p < 0.05$). Nutrients independently associated with SBP and DBP were then linearly combined into an SBP and DBP nutrient score, respectively.

We further investigated the role of dietary indices on BP using linear mixed model adjusting for age, sex, BMI, family relatedness and multiple testing in order to look at the effect of diet as a whole. For the NOVA system, subjects were stratified into tertiles of intakes for each level of processing, to determine disparities of associations between strata using the same linear mixed models.

Heritability

Taking advantage of the twin nature of our data, we estimated heritability of nutrient intakes and BP using structural equation modelling to decompose the observed phenotypic variance into three latent sources of variation: additive genetic variance (A), shared/common environmental variance (C) and non-shared/unique environmental variance (E) [25]. Additive genetic influences are indicated when monozygotic (MZ) twins are more similar than dizygotic (DZ) twins. Heritability is defined as the proportion of the phenotypic variation attributable to genetic factors, and is given by the equation, $h^2 = (A)/(A + C + E)$. The Akaike information criterion (AIC) was used to determine the best-fitting model (among ACE, AE and CE models). The model with the lowest AIC reflects the best balance of goodness of fit and parsimony [25]. The maximum likelihood method of model fitting was applied to the raw data using the R package MET.

We further investigated whether SBP/DBP share underlying genetics factors with the SBP/DBP nutrient scores. We used bivariate genetic model employing Cholesky decomposition and psychometric common pathway model [26,27].

3. Results

The demographic characteristics of the study population are presented in Table 1. A total of 3889 individuals from TwinsUK with BP measures and estimated nutrient intakes were included in the analysis. Of these, 1326 were MZ pairs, 1946 DZ pairs and 617 singletons. The study sample was predominantly female (97.9%), had mean age 54.9(12.8) years, and slightly overweight (BMI = 25.32(4.41) Kg/m^2). See Table 1.

Table 1. Characteristics of the study population (n = 3889).

Phenotype	***n***	**%**	
N	3889		
Female, *n* (%)	3808	97.2	
MZ pairs	1326	34.1	
	Mean	**SD**	
Age, yrs.	54.9	12.8	
BMI, kg/m^2	25.3	4.4	
SBP, mmHg	121.3	16.1	
DBP, mmHg	76	10.5	
Nutrients *	**Mean**	**SD**	**% energy**
Water, g	2719.24	780.97	
Alcohol, g	9.54	13.56	4.43
Carbohydrates, g	247.97	77.8	51.16
Starch, g	121.49 g	44.09	25.07
Total sugars, g	123.82	45.92	25.55
Sucrose, g	48.56	22.1	10.02
Maltose, g	3.8	2.27	0.58
Lactose, g	20.27	10.6	4.18
Saturated fats, g	25.47	10.43	11.82
Potassium, mg	3920	1058.97	
Calcium, mg	1096.2	386.02	
Magnesium, mg	340.48	96.4	
Phosphorus, mg	1494.38	421.83	
Iodine, µg	214.46	82.34	
Riboflavin, mg	2.38	0.88	
Tryptophan, mg	17.06	4.76	
Biotin, µg	47.39	14.7	

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. * Only nutrients associated with SBP and DBP after adjusting for covariates and multiple testing are included in Table 1. The full list of the nutrients included is provided in Table S2.

3.1. Nutrient Intake-Blood Pressure Associations

In the discovery cohort, out of the 45 nutrients, we found 17 nutrients associated with SBP, and 10 of those also associated with DBP after adjusting for age, sex, BMI, total energy intake, family relatedness and multiple testing using the Benjamini–Hochberg correction (FDR < 0.05) (see Table S3).

We then validated our results in the MZ discordant groups, after identifying between 17 and 242 twin pairs discordant for nutrient intakes. A total of 15 nutrients were associated with SBP and 9 also with DBP in the MZ discordant group (Figure 1). We further combined the results from the discovery and replication datasets using inverse variance fixed effect meta-analysis and found that all successfully replicated nutrient-BP associations were significant after meta-analysis (FDR < 0.05) (Figure 1).

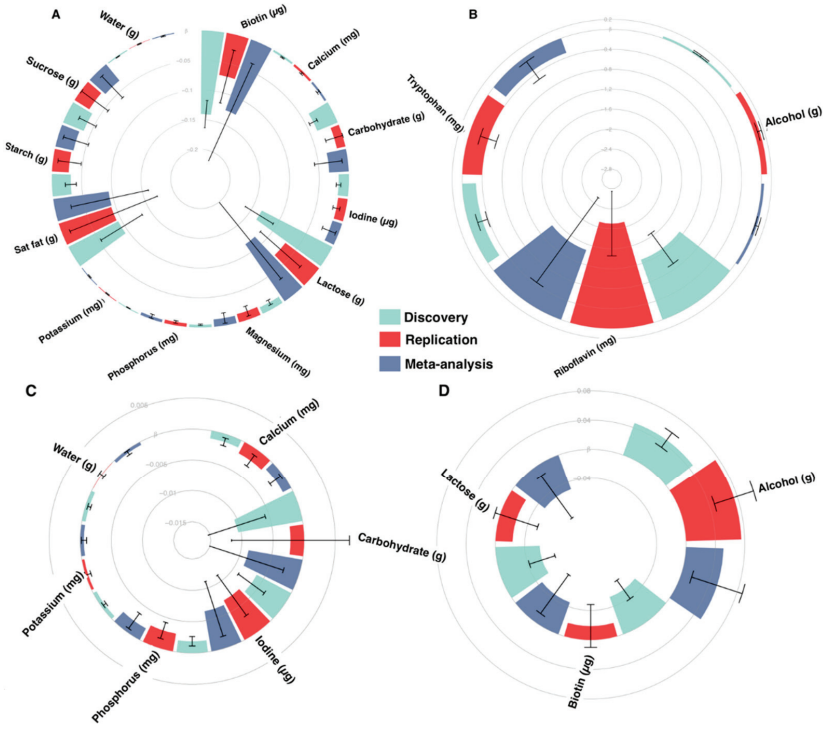

Figure 1. Meta-analysed nutrient associations with blood pressure. Nutrient-BP associations from the discovery population (False Discovery Rate (FDR) < 0.05), replication population (P < 0.05 or same direction beta) and meta-analysis. Because of the scale and comparative differences of effects, panels display varying effect sizes to facilitate visualisation of effects. SBP associations are illustrated in panels (**A**) (Beta: −0.2 to 0) and (**B**) (Beta: −2.8 to 0.2), and DBP associations are illustrated in panel (**C**) (Beta: −0.015 to 0.005) and (**D**) (Beta: −0.04 to 0.08). Error bars display SE. Results from the discovery cohort are represented in teal, from the replication cohort in red and from meta-analyses in blue.

To identify nutrients independently associated with BP, we linearly combined the significantly associated nutrients in the whole population by running a backwards stepwise regression including age, BMI, sex and family structure. Six nutrients were independently associated with SBP. These include alcohol, water, saturated fatty acid, riboflavin, tryptophan and biotin, together explaining 22.2% of the variance.

$$\begin{aligned} \mathrm{SBP}_{\mathrm{score}} = \; & 179.072827 + (0.0716177 \times \mathrm{alcohol}) - (0.0007501 \times \mathrm{water}) \\ & -(0.0968079 \times \mathrm{saturated\ fats}) + (0.0702823 \times \mathrm{riboflavin}) \\ & -(0.0150996 \times \mathrm{tryptophan}) - (0.1110900 \times \mathrm{biotin}) \end{aligned} \quad (1)$$

Three nutrients were independently associated with DBP, explaining 13.6% of variance, they include alcohol, carbohydrates and biotin:

$$\mathrm{DBP}_{\mathrm{score}} = 49.956227 + (0.070326 \times \mathrm{alcohol}) + (0.009522 \times \mathrm{carbohydrates}) - (0.047617 \times \mathrm{biotin}) \quad (2)$$

3.2. Dietary Indices and Blood Pressure

We estimated the influence of nine dietary indices and both SBP/DBP and observed no associations between any of the dietary indices and neither SBP nor DBP (Table S4). No significant associations were found also when we stratified the population by intake using the NOVA classification system.

3.3. Heritability

We estimated heritability of the 15 BP-associated nutrient using structural equation modelling and found that the best fitting model for 14 of those was the AE model with heritability estimates ranging from 27.1% [21.3%; 33%] for tryptophan to 52.7% [48.1%; 57.3%] for carbohydrates (Figure 2; Table S5). Iodine, on the other hand, appeared to be only environmentally determined.

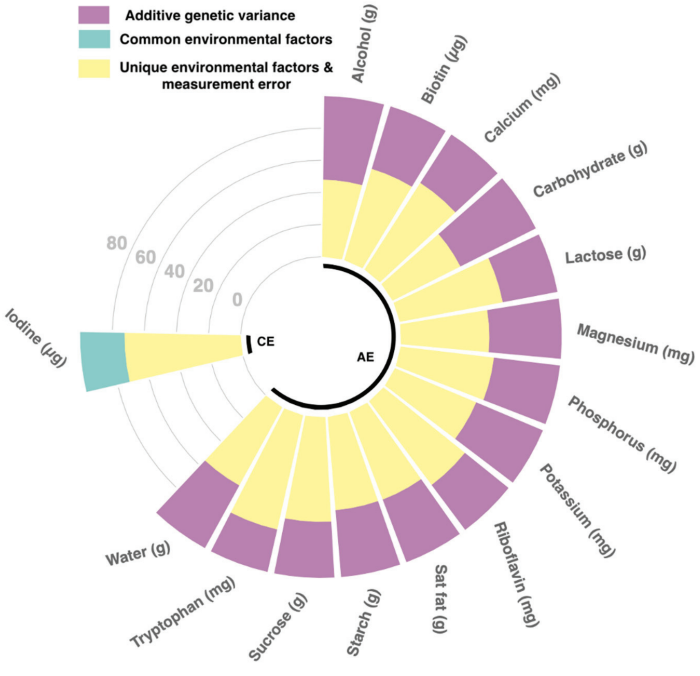

Figure 2. Circus plot of heritability for significantly associated nutrients. Plot of heritability analysis depicting sources of phenotypic variation for the 15 nutrients significantly associated with blood pressure. Nutrients in group AE were genetically derived, whereas nutrients in group CE were environmentally determined (model lowest AIC). Purple bars represent additive genetic variance, teal represent common environmental factors and yellow specific environmental factors & error.

The best fitting model for SBP/DBP scores was the AE model, with heritability estimates of 54% [49%; 58%] and 58% [53%; 62%], respectively (Table S6).

Previous studies by us and others have found SBP/DBP to have strong heritable component [28]. In this data, in line with previous findings, the best fitting model for both SBP and DBP was the AE model with heritability estimates of 53.7% for SBP and 57.6% for DBP.

We further investigated how much of the BP heritability is common to nutrient intakes (estimated with the SBP and DBP dietary scores) and found a shared heritability of 31.6% for SBP and of 30% for DBP.

4. Discussion

In one of the most comprehensive studies, incorporating 45 nutrients, 9 dietary indices and heritability to investigate diet-BP associations, we identified and replicated 15 nutrients to be associated with SBP and 9 with DBP. We also generated a nutrient score for both SBP and DBP from independently associated nutrients that, respectively, explained 22.2% and 13.6% of the variance in SBP/DBP. Both of which were positively associated with SBP and DBP respectively. Furthermore, we found that 14 out of the 15 unique nutrients were genetically determined, with heritability ranging from 27.1% to 52.7%. This is consistent with previous reports on macro- and micro-nutrients heritability ranging from 21 to 55% [29,30].

Additionally, in line with our previous results [28], we find that both SBP and DBP are heritable (h^2 = 53.7% and 57.6% respectively). Here we also report that BP and nutrients share 31.6% and 30% of the genetic influence.

The BP associated nutrients included a mix of macronutrients, amino-acids, vitamins and minerals highlighting the need to look beyond single nutrients when exploring the impact of diet on BP. The largest beneficial effects were observed for B vitamins, riboflavin (vitamin B2) for SBP and biotin (vitamin B7) for DBP.

Nutrients Independently Associated with BP

We identified six nutrients independently associated with SBP; this included alcohol, water, saturated fatty acids, riboflavin, tryptophan and biotin, which explained 22.2% of the variance. Three of those nutrients were also independently associated with DBP; these were, alcohol, carbohydrates and biotin, explaining 13.6% of variance. The percentage of variance explained by nutrients is much higher than that explained for instance by genetic factors. Indeed, over 1477 common single nucleotide polymorphisms associated with BP explain 5.7% of population phenotypic variance in SBP [5,31].

Of the six independently associated nutrient intakes, alcohol, tryptophan and riboflavin elicited large effects, in line with previously reported literature [32–40].

RIBOFLAVIN: Riboflavin, also known as vitamin B_2, is a water-soluble vitamin found in food, predominantly milk and egg products [41], and also used as a dietary supplement. The effect of riboflavin in reducing BP has been previously reported [32]. Riboflavin acts on BP in a gene-nutrient interaction involving the gene encoding methylenetetrahydrofolate reductase [33], potentially stabilising variants within this gene to restore 5-methyltetrahydrofolate concentrations to improve nitric oxide bioavailability, a potent vasodilator in BP control [32].

In our sample, riboflavin intake was above that of the average UK intake [42] (2.38 mg and 1.59 mg respectively). Here we find that the effect of riboflavin on SBP is independent from that of the other nutrients, highlighting the value of riboflavin intake for blood pressure control. Moreover, we report that riboflavin is moderately heritable, suggesting more than a third of the observed individual differences in riboflavin intake we observed, may be attributable to genetic individual differences, while the remaining 65% is due to the environment.

ALCOHOL: The present findings also illustrated that alcohol exerted the greatest deleterious effect upon both SBP and DBP, despite the average alcohol intake of our sample (Table 1) being below the average UK intake (9.54 g/d and 12.4 g/day, respectively) [43]. This is ubiquitous with that of numerous other studies [34–37,44]. Pajak and colleagues reported a strong effect for alcohol consumption and both SBP and DBP, where even low volume and frequency exerted a deleterious effect [36]. Literature repeatedly reports a J-shaped association between alcohol intake and CVD outcomes [45], but disaggregation suggests a linear-association with SBP [45]. This linear dose-response relationship was reported to exert the strongest effect in females [37]. This increased susceptibility of alcohol upon blood pressure in females may have been an observation within our female dominant cohort.

TRYPTOPHAN: Tryptophan, an essential amino acid, is commonly derived from meat products and soybeans [46]. Tryptophan is a precursor of serotonin synthesis and shown to reduce BP [47].

Serotonin is a monoaminergic neurotransmitter influencing vasoconstriction, yet the exact mechanisms underlying the relationship between tryptophan, serotonin and reduced BP are not known [48].

The association between tryptophan and BP is controversial, with some studies reporting negative correlations [49], and others, as in our study, identifying associations between tryptophan levels and decreased SBP [38–40]. This is speculated to be relating to differing dietary sources (animal versus plant) [50]. Moreover, tryptophan-containing peptides derived from the enzymatic hydrolysis of dietary protein are thought to interfere with the renin-angiotensin axis by inhibiting the rate-limiting, angiotensin converting enzyme, thereby mitigating BP [50].

These inconsistent results may also be the result of inter-individuality in sympathetic nervous system activity [51], which is the proposed mechanism in which tryptophan attenuates BP [52].

Here we report a strong negative correlation between tryptophan and SBP, supporting the role of tryptophan in BP control. Tryptophan is naturally available from animal and plant proteins [53], but increasing the consumption of a single amino-acid naturally is unfeasible. Suggesting that administration and continuous treatment of tryptophan may improve BP [48]. As tryptophan seems safe to consume, the potential health benefits has led to plant molecular genetic engineering endeavours to generate high tryptophan cereals and legumes [46].

LACTOSE: We found lactose intake to be associated to lower DBP in the univariate analysis, though lactose was not independently associated with DBP in the multivariate model. This is in line with previous studies reporting dairy products to have a lowering effect on BP [54] and lactose to have a stronger effect compared to calcium or phosphorus [54].

We also note that we did not observe any association between SBP/DBP and sodium intake [55]. This is probably due to limitations of the FFQ in estimating true sodium intake [56].

Interestingly, we observed no association between any of the dietary indices, while we found strong associations with single nutrients, highlighting the complexity of dietary patterns and underscoring how dietary indices may overlook some minor effects elicited by nutrients [9]. Dietary indices typically define dietary components that are considered important for the goal of that index, thence limiting the utility of that index [57]. Ultra-processed foods have received much attention recently in relation to health and disease [58]. In our study, when stratifying the population based upon the NOVA classification system, we detected no major differences between associations with BP in any of the NOVA strata. This novel finding is counterintuitive to our current beliefs on processed foods [18], reiterating the specificity of the index, whereby nutritional value is overlooked.

Although nutritional research is now moving away from a single nutrient approach and is focused on studying whole foods and dietary patterns, the present study underscores the value of nutrient focused dietary research in preventing and managing hypertension and our study is strengthened by numerous factors. Firstly, the large sample size facilitates the comprehensive analysis of numerous nutrients and retains sufficient sample size in the MZ replication groups. Secondly, the co-twin control study design results in the replication samples being matched for both measured and unmeasured factors, strengthening causal inferences [59]. Additionally, the use of co-twins provides the most matched genetic controls feasible [60], facilitating detrimental environmental agents of BP to be explored. Thirdly, BP was measured by experienced research nurses, whereby the first measurement was discarded and an average of two remaining measurements was used, strengthening the accuracy and precision of measurements.

We also noted some study limitations. First, findings were based upon estimated intakes generated from FFQ, limiting reliability and reproducibility as these are prone to reporting bias and random error [61,62]. Second, our study sample is predominantly female, so results may not be generalizable to men. Third, we are unable to infer causality from this cross-sectional research, requiring future interventions. This future work should investigate the mechanisms by which nutrients influence blood pressure pathways and the inter-individual differences in physiological responses to nutrients with clinical trials.

Our findings confirm current understanding, highlights the utility of nutrient focussed research to control BP and directs attention to the potential use of nutrient supplemented foods for BP control.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/7/2130/s1, Table S1: Description of dietary indices, Table S2: Descriptive nutrient intakes of TwinsUK sample (*n=3889*). Mean(SD) are provided, Table S3: Nutrient effects on SBP and DBP adjusted for covariates, Table S4: Dietary index's effects on SBP and DBP adjusted for covariates, Table S5: Heritability estimates of nutrient intakes adjusted for covariates, Table S6: Heritability estimates of blood pressure adjusted for covariates.

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A Three-Month Consumption of Eggs Enriched with ω-3, ω-5 and ω-7 Polyunsaturated Fatty Acids Significantly Decreases the Waist Circumference of Subjects at Risk of Developing Metabolic Syndrome: A Double-Blind Randomized Controlled Trial

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Abstract: Alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA), and punicic acid (PunA) are claimed to influence several physiological functions including insulin sensitivity, lipid metabolism and inflammatory processes. In this double-blind randomized controlled trial, we investigated the combined effect of ALA, DHA, RmA and PunA on subjects at risk of developing metabolic syndrome. Twenty-four women and men were randomly assigned to two groups. Each day, they consumed two eggs enriched with oleic acid (control group) or enriched with ALA, DHA, RmA, and PunA (test group) for 3 months. The waist circumference decreased significantly (−3.17 cm; $p < 0.001$) in the test group. There were no major changes in plasma insulin and blood glucose in the two groups. The dietary treatments had no significant effect on endothelial function as measured by peripheral arterial tonometry, although erythrocyte nitrosylated hemoglobin concentrations tended to decrease. The high consumption of eggs induced significant elevations in plasma low-density lipoprotein (LDL)- and high-density lipoprotein (HDL)-cholesterol ($p < 0.001$), which did not result in any change in the LDL/HDL ratio in both groups. These results indicate that consumption of eggs enriched with ALA, DHA, RmA and PunA resulted in favorable changes in abdominal obesity without affecting other factors of the metabolic syndrome.

Keywords: Alpha-linolenic acid; docosahexaenoic acid; rumenic acid; punicic acid; enriched eggs; metabolic syndrome; waist circumference; obesity

1. Introduction

Metabolic syndrome (MetS) encompasses a complex set of interrelated physiological and biochemical disorders, including disruption of lipid and glucose metabolism associated with vascular abnormalities and a pro-inflammatory state. Its most prominent components are abdominal obesity, hypertension, hyperglycaemia, hypertriglyceridemia and low level of high-density lipoprotein (HDL)-cholesterol [1]. These metabolic disorders dramatically

increase the risk of type 2 diabetes, coronary diseases, and stroke. The International Diabetes Federation indicates that people with MetS have a fivefold greater risk of developing type 2 diabetes, and that they are three times more likely to have a heart attack or stroke and twice as likely to die from it [2]. The prevalence of MetS is estimated at around one quarter of the world's population. Although this estimate varies depending on the age, ethnicity and gender of the population studied, the metabolic alterations are aggravated by lifestyle, including inactivity and dietary factors, mainly fats and some types of carbohydrates, which are strongly suspected of inducing both their development and complications [3,4].

There has been an increased interest over the past decade in understanding the metabolism of dietary lipids and their role in health. The special attention in fats has also come from the recognition that certain fatty acids are key regulators of gene expression and metabolic processes. In the worrisome context of the escalation of cardiovascular diseases (CVD) and type 2 diabetes, many researchers have studied the impact of some fatty acids on improving MetS factors.

Several follow-up studies reported that intakes of omega-3 polyunsaturated fatty acids (n-3 PUFA), including α-linolenic acid (ALA, C18:3c9,c12,c15) and its long-chain derivative, docosahexaenoic acid (DHA, C22:6c4,c7,c10,c13,c16,c19), are strongly associated with a lower risk of MetS [5,6]. Beneficial effects on the action of insulin have also been revealed by an inverse association found between the content of n-3 PUFA in the blood and insulin resistance [7,8]. A 3-month study in obese men indicated that supplementation with rumenic acid (RmA, C18:2c9,t11), an omega-7 conjugated linoleic acid (CLA) slightly decreased insulin sensitivity without altering serum lipids, glycaemia, body mass index (BMI) and body fat [9]. In contrast, Schmitt et al. [10] reported a preponderant effect of RmA in reducing insulinemia and counteracting insulin resistance after 60 days in obese and diabetic patients receiving a diet based on products rich in ALA and DHA. This suggests that combining RmA with n-3 PUFA would lead to beneficial effects on glycaemic parameters. Furthermore, punicic acid (PunA, C18:3c9,t11,c13), an omega-5 conjugated linolenic acid (CLnA), given to mice with diet-induced obesity, prevented excess body fat and improved insulin sensitivity [11]. Up to now, the combined effects of PunA, n-3 PUFA and RmA on obesity, serum lipids and glucose metabolism have not been established in both animals and humans.

In previous work, we have developed a method to enrich eggs with ALA, DHA, RmA and PunA through hen feeding [12]. The present study aimed at determining the effect of regular consumption of these eggs in adults at risk of MetS. Several key factors of the MetS were assessed, including glycosylated hemoglobin (HbA1c), insulin and fasting glucose parameters, blood pressure, abdominal obesity, lipids, and lipoproteins. We extended our study to the analysis of vascular health indicators. These include measurements of erythrocyte nitrosylated hemoglobin and endothelial function.

2. Materials and Methods

2.1. Study Population

Participants were recruited from the population aged 35 to 75, with a waist circumference greater than 80 cm for women and 94 cm for men, and practicing fewer than two hours of physical activity per week. Eligible participants completed a telephone screening to attend a medical check-up scheduled two weeks prior to the start of the study, at the Center of Investigation in Clinical Nutrition (Louvain-la-Neuve, Belgium). Comorbidity assessment and medical history review were performed for all subjects who provided information on allergies, food intolerances, smoking, drug addiction and current medication use.

Exclusion criteria were: uncontrolled hypertension (> 160/100 mmHg), type 1 or type 2 diabetes (fasting blood sugar $\geq$ 126 mg/dL or HbA1c $\geq$ 6.5%), CVD or high risk of CVD (total cholesterol > 239 mg/dL, low-density lipoprotein (LDL)-cholesterol > 159 mg/dL, HDL-cholesterol < 40 mg/dL, triglycerides > 200 mg/dL or familial history of premature cardiovascular incident); be involved in an active weight loss program or have experienced weight loss of more than 5 kg in the past three months, receiving medication or have other

health issues that might compromise compliance with the study interventions. Pregnant, lactating and perimenopausal women with symptoms, as well as postmenopausal women for less than six months were also excluded from participating. All selected participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki, and the Ethical Committee of the Cliniques universitaires Saint-Luc (Brussels, Belgium) approved the protocol. The trial is registered in the ClinicalTrials.gov database as NCT04583657.

2.2. Study Design

Participants were randomly assigned in a 1:1 ratio to either a test group or a control group. Randomization was stratified by gender using a computer-generated list of random numbers prepared by an independent statistician. In double-blind design, the test group received daily two eggs enriched with ALA, DHA, RmA and PunA, while the control group participants were given two eggs enriched with oleic acid (OA). The eggs were meant to be eaten cooked, preferably at breakfast or lunch, for three consecutive months. All eggs were produced at the University Farm of UCLouvain (Corroy-le-Grand, Belgium). The fatty acid profile of the eggs (Table 1) was checked weekly as described previously [12], to ensure it was constant throughout the study. An intervention staff strictly maintained blinding by packing the eggs in similar cardboard boxes on which a unique 3-letter code assigned to each participant was printed. Neither the assessors nor the participant knew which treatment the participant received. Boxes containing 16 eggs (2 additional eggs to prevent possible loss) were delivered to participants weekly. Upon delivery, uneaten eggs from the previous week were collected and participants responded to a questionnaire asking the occurrence, nature, severity and duration of potential adverse events, their link with the study, and whether participants had changed medication or taken a particular drug.

Table 1. Composition of fatty acids, total lipid, and cholesterol content of eggs.

	Control	**Test**
Fatty acids (mg/egg)		
Lauric acid (C12:0)	3.93 ± 0.08	3.95 ± 0.08
Myristic acid (C14:0)	9.47 ± 0.21	12.04 ± 0.28
Palmitic acid (C16:0)	993.91 ± 17.29	1008.68 ± 16.67
Palmitoleic acid (C16:1c9)	56.24 ± 2.00	51.03 ± 1.81
Stearic acid (C18:0)	344.47 ± 5.90	386.41 ± 5.52
Oleic acid (C18:1c9)	2689.32 ± 33.29	1115.25 ± 25.97
Cis-vaccenic acid (C18:1c11)	70.96 ± 1.25	35.58 ± 1.04
Linoleic acid (C18:2c9,c12)	543.01 ± 10.58	622.70 ± 13.30
Gamma-linolenic acid (C18:3c6,c9,c12)	3.52 ± 0.13	4.20 ± 0.13
Alpha-linolenic acid (C18:3c9,c12,c15)	14.31 ± 0.34	105.19 ± 4.04
Rumenic acid (C18:2c9,t11)	2.06 ± 0.21	595.16 ± 10.64
Punicic acid (C18:3c9,t11,c13)	ND	321.59 ± 9.25
Dihomo-gamma-linolenic acid (C20:3c8,c11,c14)	6.50 ± 0.13	7.55 ± 0.17
Arachidonic acid (C20:4c5,c8,c11,c14)	97.89 ± 1.46	62.40 ± 1.45
Eicosapentaenoic acid (C20:5c5,c8,c11,c14,c17)	0.05 ± 0.02	2.58 ± 0.11
n-6 Docosapentaenoic acid (C22:5c4,c7,c10,c13,c16)	23.35 ± 0.70	4.47 ± 0.24
Docosahexaenoic acid (C22:6c4,c7,c10,c13,c16,c19)	37.72 ± 0.69	82.81 ± 1.89
Σ SFA	1378.63 ± 22.40	1445.70 ± 22.31
Σ MUFA	2833.56 ± 35.64	1221.60 ± 28.01
Σ n-6 PUFA	691.28 ± 119.13	716.03 ± 138.72
Σ n-3 PUFA	61.55 ± 1.02	230.16 ± 6.15
Total lipids (% by weight of fresh yolk)	31.08 ± 0.59	32.45 ± 0.44
Total cholesterol (mg/egg)	177.19 ± 5.40	182.26 ± 10.13

Values as "mean ± SEM". ND: not detected; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; n-6 PUFA: omega-6 polyunsaturated fatty acids; n-3 PUFA: omega-3 polyunsaturated fatty acids.

The three-month trial period included four medical visits: one at the start of the study (month 0 or baseline), when the subjects received the eggs for the first time, and

three monthly visits (months 1, 2 and 3), the last of which ended the study. The primary endpoint chosen for the design of the study was the mean change in HbA1c from baseline to month 3. Other efficacy endpoints included obesity, serum lipids, insulin sensitivity, inflammation, and endothelial function. All assessments, except endothelial function, were performed at each monthly visit. The participants were requested not to change their habits regarding physical activity, to eat fish no more than twice a week, and to abstain from dietary supplements of n-3 PUFA, CLA or CLnA during the trial commitment period. The study was conducted from June 2019 to March 2020.

2.3. Dietary Assessment

Prior to medical visit, the subjects performed a 3-day dietary record. Standard food servings and a food atlas [13] were used to quantify household measures. The macronutrient composition of the diets was assessed using the French Agency for Food, Environmental and Occupational Health Safety database (ANSES-CIQUAL).

2.4. Anthropometric Measurements

Body weight was determined to the nearest 0.1 kg, height, waist, and hip circumferences to the nearest 0.1 cm, in subjects wearing light indoor clothing and without shoes. The waist-to-hip ratio (WHR) was calculated from these measurements. BMI was calculated by dividing the body weight in kilograms by the square of the height in meters. Body fat and lean body mass were calculated using a Tanita SC-240 bioelectric impedance analyzer (Tanita Europe BV, Amsterdam, The Netherlands) and manufacturer's programmed equations.

2.5. Clinical Investigations

Blood samples were obtained from subjects after a 10- to 12-h overnight fast. Triglycerides, HDL-, non-HDL- and LDL-cholesterol, glycaemia, insulinemia, homeostasis model assessment for insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI), HbA1c, hemoglobin, hematocrit, erythrocytes, leucocytes, thrombocytes, high-sensitivity C-reactive protein (hs-CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (GGT), urea, creatinine, estimated glomerular filtration rate (eGFR) and albumin were analyzed at the Clinique St Pierre (Ottignies, Belgium).

IL-6 and TNF-α assays were performed using Human IL-6 and TNF-α Quantikine HS ELISA kits (R&D Systems, Langley, UK) respectively, and oxidized LDL (ox-LDL) were measured employing a Mercodia oxidized LDL ELISA kit (Mercodia, Huissen, The Netherlands), according to manufacturer's instructions.

2.6. Measurement of Nitrosylated Hemoglobin and Peripheral Artery Tonometry

On two occasions, at the first (month 0) and the last visit (month 3), the endothelial function was assessed by peripheral arterial tonometry and nitrosylated hemoglobin measurement.

Blood was drawn by venopuncture from the median cubital vein into a vacutainer tube containing EDTA (K2E, Vacutainer, BD-Plymouth, UK). A mixture of antioxidant solution (sodium ascorbate and N-acetylcysteine; final concentration, 5 mmol/L of both) was added into closed vacutainer using a Micro-Fine™ syringe prior the centrifugation to support the blood redox condition. The erythrocytes were collected after centrifugation (10 min, 800× *g*, at room temperature) from the bottom of the vacutainer tube into a 1 mL syringe and stored immediately at −80 °C. The concentration of heme-(Fe II)-nitrosyl-hemoglobin (HbNO) was assessed in the erythrocyte samples using the low-temperature Electron Paramagnetic Spectroscopy (EPR). The EPR spectra were recorded by an X-band EPR spectrometer (EMX-micro) (Bruker Instruments Inc., Billerica, MA, USA) with the following settings: microwave frequency, ~ 9.35 GHz; modulation frequency, 100 kHz; microwave power, 20 mW; modulation amplitude, 0.7 mT at 77 K using an EPR quartzfinger Dewar filled with liquid nitrogen. The erythrocyte concentration of the HbNO complex (5-

coordinate α-heme-nitrosyl) was quantified from the intensity of the hyperfine components of the HbNO EPR signal (g-factor 2.01, Ahf = 16.8 G) after subtraction of the overlapping EPR signal of protein free radicals from the integral EPR spectrum of frozen erythrocytes following the method developed previously [14].

Endothelial vasodilation function was determined using the Endo-PAT 2000 device (Itamar Medical, Caesarea, Israel). Subjects were directed to rest in a quiet, dimly lit, temperature-controlled exam room. Two high-sensitive pneumatic probes (EndoPAT™, Itamar) were placed on the index fingers of the left and right hands, and plethysmographic signals in the index fingers were recorded throughout the test. A baseline record was performed for five minutes. For the 5-min occlusion phase, the blood pressure cuff placed on the right forearm was inflated to a supra-systolic pressure of 60 mmHg above the patient's systolic pressure or to 200 mmHg, depending on the greater value between the two. Complete cessation of blood flow to the hand was verified by the absence of a peripheral arterial tone (PAT) signal from the occluded arm. Then the cuff was abruptly deflated as quickly as possible to initiate the post-occlusion recording phase (5 min). The reactive hyperemia index (RHI) was calculated by the device as the ratio of the post-to-pre-occlusion PAT signal in the ischemic arm (right arm), relative to the same ratio in the control arm (left arm), and corrected for baseline vascular tone [15]. Arterial stiffness was assessed by the augmentation index (AI), which was determined from the baseline resting pulse wave.

2.7. Statistics and Data Analysis

The study was designed to show a statistically significant 10% decrease in the primary endpoint (HbA1c) in the test group assuming a standard deviation of 0.8, using a two-sample t-test with 80% power and a 5% level of significance. PASS 14.0.7 software (NCSS, Kaysville, UT, USA) was used for the calculation based on 40 subjects per group and a dropout rate of 10%. Because of the lockdown measures intended to limit the spread of cases of covid-19 contamination from March 2020 on, the trial had to be stopped after 24 subjects had completed the experimental period.

Statistical analyses were performed using the software systems SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and JMP Pro 15 SAS. Changes between groups (control and test) per visit (1, 2, 3 and 4) and between visits 1 and 4 for each group were analyzed using a linear mixed model for repeated measurements with subjects as random variable, and groups, visits, and their interaction as fixed independent variables. When the interaction was significant (p-value < 5%), pairwise comparisons were computed using t-tests followed by Bonferroni correction on selected combinations of groups and visits. When there was no significant interaction but the difference between groups or visits was significant, pairwise comparisons using Tukey's test were computed. If necessary, a logarithm to the base 10 was used to fulfill the assumptions of the mixed model. Variables that were not normally distributed after the logarithmic transformation were analyzed using non-parametric methods. For analyses of within-group differences, the Wilcoxon matched-pairs signed rank test was used. The Wilcoxon Mann-Whitney two-sample test was used for analyses of differences between groups.

3. Results

3.1. Dietary Analysis and Tolerance

Twenty-four subjects, 12 in each treatment group, were included in the study as shown in Figure 1 and Table 2. All participants completed the trial. Adherence to study was assessed based on the number of uneaten eggs and the dietary record provided by the participants. The estimate of eggs consumed over those prescribed was greater than 95% in all participants.

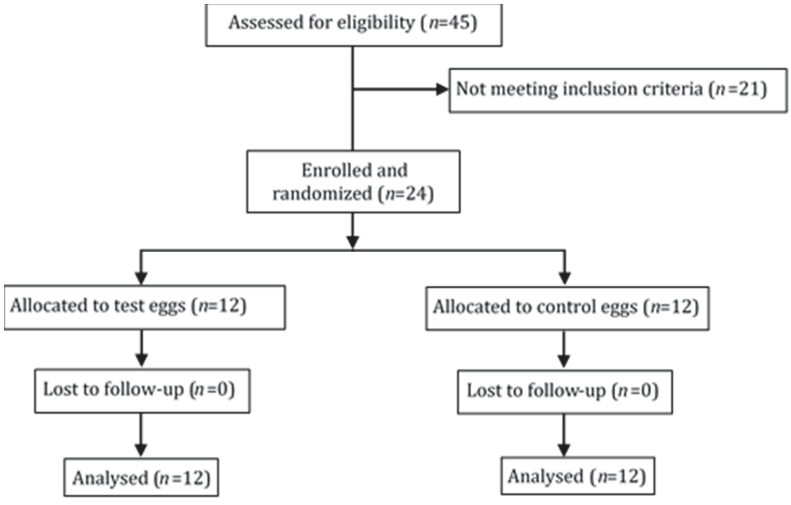

Figure 1. Recruitment flow diagram.

Table 2. Baseline characteristics of participants in control and test groups.

	Control (n = 12)		Test (n = 12)		p-Value [a]
	Mean ± SEM	Range	Mean ± SEM	Range	
Sex (women/men)	9/3	-	8/4	-	-
Age (years)	51.67 ± 2.39	42–71	45.58 ± 2.43	35–59	0.105
Body weight (kg)	76.23 ± 2.64	66.4–93.8	74.34 ± 4.18	59.1–98.1	0.402
BMI (kg/m^2)	25.98 ± 0.75	21.9–30.3	26.04 ± 1.20	20.3–35.6	0.402
Waist circumference (cm)	93.38 ± 2.69	82–111	92.21 ± 2.79	83–111	0.840
Hip circumference (cm)	106.38 ± 1.49	99–115	102.79 ± 2.91	92–132	0.296
WHR	0.88 ± 0.03	0.8–1.0	0.89 ± 0.02	0.8–1.1	0.564
Body fat (%)	33.48 ± 1.71	22.5–42.3	31.83 ± 2.24	18.1–48	0.624
Lean body mass (kg)	50.13 ± 2.56	42.7–66.6	49.81 ± 2.65	37.4–64.9	1.000
Hemoglobin (g/dL)	13.89 ± 0.34	12.2–15.5	14.18 ± 0.49	12–16.71	0.751
Hematocrit (%)	41.6 ± 0.89	36.1–46.4	42.20 ± 1.24	36.2–48.2	0.773
Red blood cells (10^6/mm^3)	4.66 ± 0.10	4.08–5.22	4.75 ± 0.16	3.93–5.66	0.977
White blood cells (10^3/mm^3)	5.28 ± 0.27	3.95–7.13	5.52 ± 0.26	3.93–7.27	0.507
Platelets (10^3/mm^3)	226 ± 14.09	144–301	222.17 ± 15.07	144–301	0.665
AST (UI/L)	18.83 ± 1.07	14–27	21.64 ± 2.13	14–35	0.477
ALT (UI/L)	17.25 ± 2.07	10–34	20.67 ± 2.69	10–44	0.247
GGT (UI/L)	15.50 ± 0.97	10–20	18.17 ± 2.52	9–36	0.664
Urea (mg/dL)	30.75 ± 1.50	20–38	33 ± 2.17	23–50	0.401
Creatininemia (mg/dL)	0.79 ± 0.02	0.67–0.93	0.86 ± 0.04	0.67–1.09	0.247
eGFR (mL/min)	92.42 ± 2.28	82–104	90.75 ± 3.35	73–112	0.623
Albumin (g/dL)	4.30 ± 0.09	3.90–4.90	4.31 ± 0.06	3.90–4.60	0.640
Total protein (g/dL)	6.94 ± 0.14	6–7.50	6.78 ± 0.12	6.20–7.30	0.271

n = number of subjects. [a] Differences between the two groups were analyzed using Wilcoxon Mann-Whitney two-sample test (significant at p-value < 0.05). BMI: body mass index; WHR: waist-to-hip ratio; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; eGFR: estimated glomerular filtration rate.

Dietary analysis based on four 3-day food records, one completed before and the others completed during the study, showed that participants did not change their dietary intake with regard to energy, protein, carbohydrates, and fat (Table 3). Besides, the average daily intakes of energy and macronutrients did not differ significantly between groups. DHA intake increased in the test group. However, the difference in the DHA content of the control and the test eggs did not allow reaching a significant difference in the average

daily intake of this n-3 PUFA between the two groups. In contrast, the consumption of approximately 1190 mg/day of RmA and 643 mg/day of PunA through the test eggs allowed a significant increase ($p < 0.001$) of their intakes by the test group. Subjects in both groups doubled their cholesterol intake during the study.

Table 3. Dietary intake of study participants based on the 3-day food record.

	Months			
	0	**1**	**2**	**3**
Energy (kcal/day)				
Control	1865.2 ± 159.06 ^{a}	1811.92 ± 146.90 ^{a}	1805.75 ± 145.23 ^{a}	1818.33 ± 161.42 ^{a}
Test	1782.45 ± 100.32 ^{a}	1531.36 ± 81.53 ^{a}	1637.36 ± 105.59 ^{a}	1538.27 ± 137.10 ^{a}
Protein (% of daily energy)				
Control	16.00 ± 0.93 ^{a}	17.67 ± 1.24 ^{a}	17.92 ± 1.12 ^{a}	17.50 ± 0.72 ^{a}
Test	15.18 ± 1.23 ^{a}	17.27 ± 1.15 ^{a}	15.91 ± 0.94 ^{a}	18.55 ± 1.67 ^{a}
Carbohydrates (% of daily energy)				
Control	43.50 ± 1.92 ^{a}	38.67 ± 1.31 ^{a}	40.75 ± 1.72 ^{a}	40.33 ± 1.68 ^{a}
Test	38.27 ± 2.29 ^{a}	39.45 ± 2.18 ^{a}	36.64 ± 2.13 ^{a}	36.09 ± 2.02 ^{a}
Fiber (g/day)				
Control	21.4 ± 1.54 $^{a\#}$	20.00 ± 16.77 $^{a\#}$	19.17 ± 15.67 $^{a\#}$	20.58 ± 1.56 $^{a\#}$
Test	16.45 ± 1.77 $^{a\#}$	14.18 ± 1.32 $^{a\#}$	14.09 ± 1.47 $^{a\#}$	14.09 ± 135 $^{a\#}$
Lipids (% of daily energy)				
Control	35.62 ± 2.49 ^{a}	36.79 ± 1.74 ^{a}	36.19 ± 1.71 ^{a}	36.06 ± 1.73 ^{a}
Test	38.08 ± 2.19 ^{a}	36.58 ± 2.02 ^{a}	40.90 ± 2.17 ^{a}	40.09 ± 1.99 ^{a}
SFA (% of daily energy)				
Control	16.44 ± 1.25 ^{a}	14.80 ± 1.05 ^{a}	14.63 ± 1.22 ^{a}	14.25 ± 0.82 ^{a}
Test	15.22 ± 0.88 ^{a}	15.82 ± 1.42 ^{a}	17.59 ± 1.15 ^{a}	16.81 ± 0.83 ^{a}
MUFA (% of daily energy)				
Control	10.29 ± 0.95 ^{a}	12.70 ± 0.80 ^{b}	12.27 ± 0.80 ^{ab}	12.88 ± 0.74 ^{b}
Test	13.43 ± 1.51 ^{a}	11.40 ± 0.85 ^{a}	11.86 ± 1.43 ^{a}	11.09 ± 1.03 ^{a}
PUFA (% of daily energy)				
Control	3.40 ± 0.36 ^{a}	4.80 ± 0.45 ^{b}	4.39 ± 0.31 ^{ab}	4.58 ± 0.34 ^{b}
Test	4.27 ± 0.62 ^{a}	5.02 ± 0.31 ^{a}	5.00 ± 0.44 ^{a}	5.56 ± 0.54 ^{a}
Alpha-linolenic acid (mg/day)				
Control	593.9 ± 132.63 ^{a}	930.58 ± 136.68 ^{a}	728.33 ± 103.98 ^{a}	679.17 ± 129.75 ^{a}
Test	697.81 ± 119.61 ^{a}	675 ± 87.35 ^{a}	629.73 ± 100.52 ^{a}	737.82 ± 168.48 ^{a}
Rumenic acid (mg/day)				
Control	125.9 ± 19.23 ^{a}	116.08 ± 28.92 $^{a\#\#}$	108.75 ± 19.82 $^{a\#\#}$	117.58 ± 17.81 $^{a\#\#}$
Test	92.64 ± 19.93 ^{a}	1167.27 ± 116.94 $^{b\#\#}$	1106.55 ± 131.88 $^{b\#\#}$	1187.73 ± 112.41 $^{b\#\#}$
Punicic acid (mg/day)				
Control	0.00 ± 0.00 ^{a}	0.00 ± 0.00 $^{a\#\#\#}$	0.00 ± 0.00 $^{a\#\#\#}$	0.00 ± 0.00 $^{a\#\#\#}$
Test	0.00 ± 0.00 ^{a}	643.18 ± 18.51 $^{b\#\#\#}$	643.18 ± 18.51 $^{b\#\#\#}$	643.18 ± 18.51 $^{b\#\#\#}$
Docosahexaenoic acid (mg/day)				
Control	148.20 ± 63.85 ^{a}	216.17 ± 51.55 ^{a}	230.42 ± 59.12 ^{a}	265.50 ± 126.67 ^{a}
Test	101.55 ± 37.62 ^{a}	189.64 ± 14.02 ^{ab}	278.27 ± 72.18 ^{b}	232.73 ± 34.41 ^{b}
Cholesterol (mg/day)				
Control	284.30 ± 38.36 ^{a}	621.50 ± 34.93 ^{b}	581.08 ± 19.91 ^{b}	593.5 ± 17.38 ^{b}
Test	262.45 ± 38.25 ^{a}	571 ± 21.43 ^{b}	589.09 ± 54.26 ^{b}	573.45 ± 23.66 ^{b}

Values as "mean ± SEM". Differences between the values were determined using *t*-test. Values in the same row with no common superscripts (a, b) are significantly different, $p < 0.05$. The difference between the two groups within the same month was significant at $p < 0.05$ (#), $p < 0.001$ (##) or $p < 0.0001$(###). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Participants experienced no adverse events except that two subjects in the test group reported mild nausea at some occasions. The following clinical chemistry variables: AST, ALT, GGT, hemoglobin, hematocrit, erythrocytes, leucocytes, platelets, albumin, total serum proteins, urea, creatinine and eGFR were relatively constant and remained within normal ranges from the beginning to the end of the study in both groups (data not shown).

3.2. Vital Signs and Anthropometrics

Heart rate, and systolic and diastolic blood pressures remained relatively constant in the two groups, without differences between them (Table 4). Body weight, BMI, waist circumference, and body composition were comparable across the two groups at the baseline (Table 2). When changes in these variables were analyzed over the course of the study, they displayed opposite patterns between the control group and the test group (Figure 2). However, the differences were not statistically significant for body weight, BMI, body fat and lean mass. The changes in waist circumference and in WHR were significant ($p < 0.05$) between the control and the test groups after two months of treatment. No significant difference was found within the control group from month 0 (baseline) to month 3, while waist circumference was reduced on average by 3.17 cm in the test group ($p < 0.001$, Table 4). To further explore the waist circumference response to dietary treatments, subjects were grouped by gender (Control group: females = 9 and males = 3; Test group, females = 8 and males = 4). No gender differences could be observed in any of the groups when performing this secondary analysis. The women and men in the control group showed a mean ± SEM reduction of 0.67 ± 1.02 and 0.01 ± 1.00 cm, respectively, while the women and men in the test group experienced a reduction in their waist circumference of 3.12 ± 1.41 and 3.25 ± 2.14 cm, respectively.

Table 4. Changes in blood pressure, anthropometric measurements, glycaemic parameters and serum lipids between start and end of study.

	Control (n = 12)			Test (n = 12)		
	Month 0	**Month 3**	**Δ, Month 3-0**	**Month 0**	**Month 3**	**Δ, Month 3-0**
Resting heart rate (beats/min)	68.33 ± 2.23	66.50 ± 2.22	−1.83 ± 2.22	66.25 ± 2.51	69.50 ± 2.24	3.25 ± 2.45
Systolic blood pressure (mmHg)	128.33 ± 4.80	122.33 ± 3.17	−6.00 ± 3.01	122.33 ± 4.76	118.17 ± 5.59	−4.17 ± 4.39
Diastolic blood pressure (mmHg)	78.50 ± 3.27	76.25 ± 1.85	−2.25 ± 2.58	77.08 ± 3.44	73.33 ± 3.57	−3.75 ± 2.45
Weight (kg)	76.23 ± 2.64	76.32 ± 2.76	0.09 ± 0.58	74.34 ± 4.18	73.48 ± 4.01	−0.86 ± 0.58
BMI (kg/m^2)	25.98 ± 0.75	26.01 ± 0.79	0.03 ± 0.19	26.04 ± 1.20	25.78 ± 1.16	−0.26 ± 0.19
Waist circumference (cm) [a]	93.38 ± 2.69	93.88 ± 2.69	0.50 ± 0.79	92.21 ± 2.79	89.04 ± 2.60	−3.17 ± 1.12 **
Hip circumference (cm)	106.38 ± 1.49	105.63 ± 1.44	−0.75 ± 0.53	102.79 ± 2.91	101.83 ± 2.57	−0.96 ± 1.11
WHR	0.88 ± 0.03	0.89 ± 0.02	0.01 ± 0.01	0.90 ± 0.02	0.88 ± 0.03	−0.02 ± 0.01
Body fat (%)	33.48 ± 1.71	35.62 ± 2.30	2.14 ± 1.95	31.83 ± 2.24	29.48 ± 2.64	−2.35 ±1.37
Lean mass (kg)	50.13 ± 2.56	49.08 ± 2.43	−1.04 ± 1.64	49.81 ± 2.65	51.54 ± 3.00	1.73 ± 1.44
Fasting blood glucose (mg/dL) [a]	86.75 ± 1.44	86.42 ± 2.90	−0.33 ± 2.43	87.25 ± 2.69	89.42 ± 2.42	2.17 ± 2.77
Fasting insulin (mUI/mL) [a]	6.47 ± 0.66	5.76 ± 0.54	−0.71 ± 0.48	6.24 ± 0.82	7.38 ± 1.00	1.13 ± 0.90
HOMA-IR	1.40 ± 0.16	1.25 ± 0.14	−0.15 ± 0.12	1.38 ± 0.20	1.68 ± 0.27	0.30 ± 0.25
QUICKI	0.37 ± 0.03	0.38 ± 0.01	0.01 ± 0.004	0.38 ± 0.04	0.36 ± 0.01	−0.01 ± 0.01
HbA1c (mmol/mol) [a]	33.33 ± 1.00	34.00 ± 0.82	0.67 ± 0.31	33.33 ± 1.05	33.75 ± 1.02	0.42 ± 0.29
Triglycerides (mg/dL)	70.17 ± 5.74	70.83 ± 6.80	0.67 ± 7.29	77.25 ± 9.98	79.17 ± 11.82	1.92 ± 9.33
Total cholesterol (mg/dL)	184.67 ± 7.21	193.50 ± 7.95	8.83 ± 4.44 **	170.50 ± 6.66	192.83 ± 6.67	22.33 ± 5.46 **
LDL-cholesterol (mg/dL)	112.67 ± 6.39	118.08 ± 7.88	5.42 ± 3.60 **	100.00 ± 6.29	116.00 ± 7.06	16.00 ± 4.51 **
HDL-cholesterol (mg/dL)	57.92 ± 2.87	61.25 ± 2.92	3.33 ± 1.29 **	55.08 ± 2.70	61.00 ± 3.36	5.92 ± 1.64 **
LDL/HDL	1.99 ± 0.14	1.97 ± 0.15	−0.03 ± 0.05	1.90 ± 0.19	2.01 ± 0.20	0.10 ± 0.07
non-HDL-cholesterol (mg/dL)	126.75 ± 6.66	132.25 ± 7.38	5.50 ± 3.73 **	115.42 ± 8.09	131.83 ± 8.28	16.42 ± 4.53 **

n = number of subjects. Δ: change between the two months. Values as "mean ± SEM". BMI: body mass index; WHR: waist-to-hip ratio; HOMA-IR: homeostasis model assessment for insulin resistance; QUICKI: quantitative insulin sensitivity check index; HbA1c: glycosylated hemoglobin; LDL: low-density lipoprotein; HDL: high-density lipoprotein. [a] Variables were logarithmically transformed prior to paired t-test within groups. (**) Significant change from month 0 to 3, $p < 0.001$.

3.3. Insulin and Glucose Metabolism

Blood glucose and insulin levels did not differ in the two groups at month 3 compared to month 0. HOMA-IR and QUICKI were also not affected by the different treatments (Table 4). HbA1c levels increased significantly from month 0 to month 1 ($p < 0.01$) in the control group and from month 0 to months 1 and 2 ($p < 0.01$ and $p < 0.05$, respectively) in the test group, and then returned at month 3 to levels similar to those at month 0 (Figure 3). There was no significant difference between the two groups regarding values for the same month.

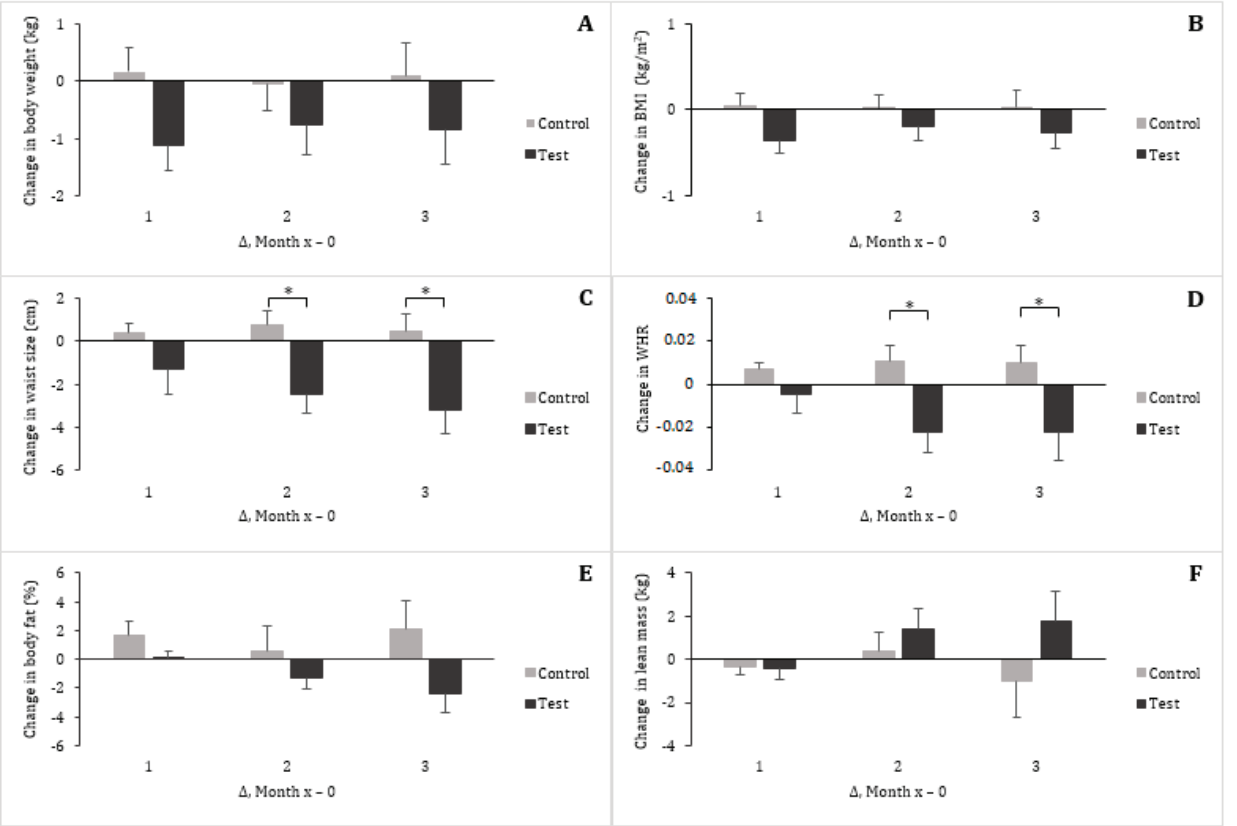

Figure 2. Changes (Δ) in body weight (**A**), body mass index (BMI) (**B**), waist circumference (**C**), waist-to-hip ratio (WHR) (**D**), percentage of body fat (**E**) and lean body mass (**F**) in men and women daily consuming two eggs enriched in oleic acid (control group) or two eggs enriched in α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) (test group), from the start of the study (Month 0) to months 1, 2 and 3 (Months x). Mean (± SEM). (*) for significant differences between control group and test group at $p < 0.05$, analyzed using *t*-test.

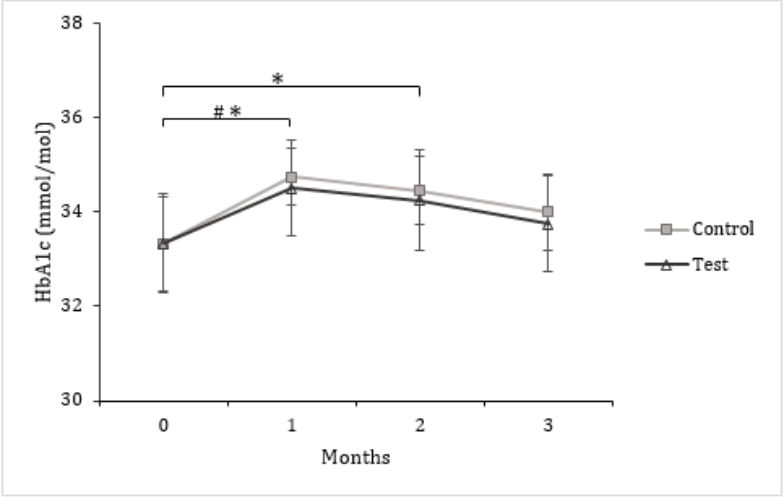

Figure 3. Glycosylated hemoglobin (HbA1c) in response to the daily consumption of two eggs enriched in oleic acid (OA) by the control group (□) or two eggs enriched in α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) by the test group (Δ), for 3 months. Mean (± SEM). Variables were logarithmically transformed prior to paired t-test, $p < 0.05$. (#) Significant difference compared to month 0 within the control group; (*) Significant difference compared to month 0 within the test group.

3.4. Serum Triglycerides and Lipoproteins

Changes in serum triglycerides were very small throughout the study and did not differ among treatment groups (Table 4). Total cholesterol concentrations were significantly increased in the two groups ($p < 0.001$), due to a significant increase in HDL and non-HDL-cholesterol ($p < 0.001$ in both groups) (Table 4, Figure 4). No difference between the groups was found. Analysis of within-group profiles showed that seven subjects in the control group had normal total cholesterol levels (<190 mg/dL) at the start of the study. Five subjects remained within normal levels, while the other two had an increase in total cholesterol above normal level by the first month of treatment. One subject in the control group with a high level at month 0 had a decrease in total cholesterol to a normal level at month 3. Concerning the test group, ten subjects had normal total cholesterol levels at month 0. Four of them exceeded the normal limit (two from month 1 and two at month 3), whereas two other subjects experienced an increase in total cholesterol above normal at months 1 and 2, then a decrease to normal levels at month 3.

3.5. Inflammation and Oxidative Stress

At month 0, there were no significant differences in plasma inflammatory markers between the groups, except that hs-CRP was significantly lower ($p < 0.05$) in the test group (Table 5). Both treatments had no effect on IL-6 and TNF-α levels. There was a within-group trend toward an increase in hs-CRP ($p < 0.08$) in the test group, while no change was observed in the control group. Plasma ox-LDL levels increased in both groups at month 3 compared to month 0. However, the increase was statistically significant only in the test group ($p < 0.05$). There was no statistically significant difference between the groups at months 0 and 3.

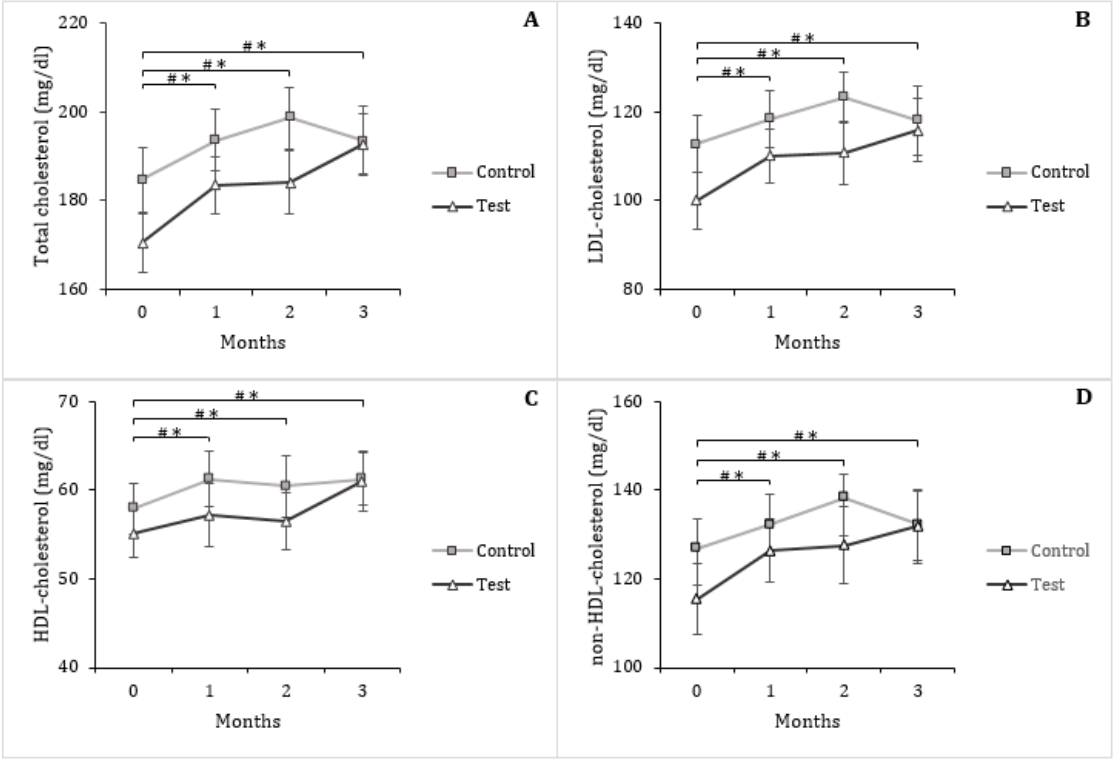

Figure 4. Total cholesterol (**A**), low-density lipoprotein (LDL)-cholesterol (**B**), high-density lipoprotein (HDL)-cholesterol (**C**) and non-HDL-cholesterol (**D**) in response to the daily consumption of two eggs enriched in oleic acid (OA) by the control group (□) or two eggs enriched in α-linolenic acid (ALA), docosahexaenoic acid (DHA), rumenic acid (RmA) and punicic acid (PunA) by the test group (Δ) during 3 months. Mean (± SEM). Paired t-test, $p < 0.001$. (#) Significant difference compared to month 0 within the control group; (*) Significant difference compared to month 0 within the test group.

Table 5. Changes in inflammation, oxidative stress and vascular health parameters between start and end of study.

	Control (n = 12)			Test (n = 12)		
	Month 0	**Month 3**	**Δ, Month 3-0**	**Month 0**	**Month 3**	**Δ, Month 3-0**
hs-CRP (mg/dL)	0.23 ± 0.06 #	0.19 ± 0.04	−0.03 ± 0.06	0.09 ± 0.02#	0.21 ± 0.06	0.11 ± 0.06
IL-6 (pg/mL)	1.29 ± 0.21	1.29 ± 0.24	0.00 ± 0.27	1.20 ± 0.16	1.69 ± 0.36	0.49 ± 0.36
TNF-α (pg/mL)	0.87 ± 0.08	0.86 ± 0.07	−0.01 ± 0.04	0.79 ± 0.05	0.85 ± 0.05	0.06 ± 0.03
Ox-LDL (U/L)	55.66 ± 4.14	59.13 ± 3.75	3.47 ± 3.51	51.89 ± 4.29 *	63.06 ± 3.94 *	11.16 ± 9.81
HbNO (nmol/L)	102.50 ± 10.37	78.44 ± 13.59	−24.06 ± 17.48	110.42 ± 18.29	83.86 ± 16.39	−26.56 ± 14.33
RHI	2.30 ± 0.18	2.17 ± 0.17	−0.18 ± 0.20	2.32 ± 0.19	1.98 ± 0.19	−0.34 ± 0.12
LnRHI	0.81 ± 0.08	0.74 ± 0.09	−0.09 ± 0.11	0.81 ± 0.08	0.63 ± 0.10	−0.17 ± 0.06
AI (%)	18.74 ± 6.20	20.50 ± 8.12	1.76 ± 4.33	12.60 ± 6.33	14.94 ± 5.66	2.33 ± 2.66
AI@75bpm (%)	8.92 ± 6.21	12.95 ± 9.01	4.03 ± 5.39	4.64 ± 5.66	7.34 ± 5.22	2.69 ± 2.53

n = number of subjects. Δ: change between the two months. Values as "mean ± SEM". (*) Significant difference within groups analyzed using Wilcoxon matched-paired signed rank test, $p < 0.05$. (#) Significant difference in the same month between the two groups analyzed using Wilcoxon Mann-Whitney two-sample test, $p < 0.05$. hs-CRP: high-sensitivity C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; Ox-LDL: oxidized low-density lipoprotein; HbNO: nitrosylated hemoglobin; RHI: reactive hyperaemia index; AI: augmentation index; AI@75bpm: augmentation index adjusted to a heart rate of 75 beats per minute.

3.6. Erythrocyte Level of Nitrosylated Hemoglobin and Vascular Endothelial Function

As shown in Table 5, erythrocyte HbNO concentrations decreased in both study groups at month 3 (−24.06 nmol/L in the control group and −26.56 nmol/L in the test group). However, these differences were not statistically significant. The reactive hyperaemia indexes (RHI and LnRHI) did not differ among groups and throughout the study. The AI measurement for arterial stiffness was not significantly altered by either condition tested.

4. Discussion

In this double-blind randomized controlled parallel-group trial, we examined the effect of combined supplementation of ALA, DHA, RmA, and PunA on MetS components in healthy women and men, but with a high propensity to develop a MetS. OA was chosen as the control for its benefits in the management of CVD [16]. Its positive effects include lowering LDL-cholesterol and increasing HDL-cholesterol [17], and it may also help in good control of hypertriglyceridemia [18]. Chicken egg was of particular interest as a dietary carrier for supplementation since it is relatively high in lipids and has great culinary versatility. The good compliance and the zero dropout rate observed during the study indicate that both control and test eggs were well tolerated by the participants.

One of the findings of this study is that the waist circumference decreased significantly in the subjects given the test eggs, while body weight, percent of body fat and lean mass were relatively unchanged (Figure 2). This suggests changes in body fat distribution and a positive effect of these eggs on abdominal obesity. In the placebo-controlled, randomized clinical trial by Defina et al. [19] where overweight and obese individuals received n-3 PUFA supplementation (3 g/day for 6 months), no difference in weight and body composition was observed compared to the placebo group. These results were confirmed by subsequent studies in subjects with CVD and type 2 diabetes [20,21]. In contrast, multiple studies showed convincing effects of CLA on reducing weight and body fat in overweight and obese humans [22–24]. The isomers of CLA that have been the most investigated so far are RmA and t10,c12-CLA (C18:2t10,c12). In a meta-analysis examining the effectiveness of CLA in reducing body fat, Whigham et al. [25] highlighted the low number of human studies conducted with a single isomer. The few existing interventions mostly agree that RmA does not have significant effect on body composition in humans [9,26]. In agreement with this, numerous animal and some human studies have shown that of the two tested isomers of CLA, t10,c12-CLA specifically is responsible for the anti-obesity effects [27–29]. The effects observed in the present study could not be attributed to t10,c12-CLA, as it was not identified in the test eggs. Similar to our results, a patented lipid-based formulation containing PunA induced a reduction of waist circumference of 1.05 cm without change in body weight, body fat and muscle mass. Unfortunately, no indication of the amount of PunA used was provided [30].

Koba et al. [31] showed that PunA administered to mice induced a reduction in adipose tissue weight accompanied by an increased in carnitine-palmitoyltransferase activity in the liver and brown adipose tissue. The authors stated that the anti-obesity effect of PunA could be in part due to the stimulation of β-oxidation of fatty acids [32]. Furthermore, Lai et al. [33] found in 3T3-L1 cells that PunA decreased adipogenesis and preadipocyte differentiation by down-regulating the levels of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein (C/EBP)β and C/EBPδ, as well as fatty acid synthase, a key enzyme in lipogenesis.

Fasting blood sugar and insulin remained unchanged in both groups throughout the study, as did HOMA and QUICKI since these indices are derived from blood sugar and insulinemia values. The HbA1c value provides information about the average concentration of glucose in the blood over the 2 to 3 months preceding the test. Surprisingly, the levels of HbA1c increased after one month of treatment before returning to baseline values at the end of the study (Figure 3). The reason of this transient modulation has not been identified. Since blood sugar, insulin and HbA1c were globally not affected between the start and the end of the study, we can state that the combined supplementation of ALA,

DHA, RmA and PunA through egg consumption for 3 months has neither diabetogenic nor diabeto-mitigating effects.

One whole egg provides on average 177.19 mg (control egg) or 182.26 mg (test egg) of cholesterol (Table 1). Consequently, both groups reported a significant elevation in dietary cholesterol during the study period. The high blood cholesterol levels observed in both groups might therefore be attributed to the eggs consumed. Contradictorily, Fuller et al. [34], in a 3-month study in overweight or obese people with prediabetes or type 2 diabetes, found no significant difference in the change in triglycerides, total, LDL- and HDL-cholesterol between people consuming a high-egg diet ($\geq$12 eggs/week) compared with those consuming a low-egg diet (<2 eggs/week). The same research group subsequently showed that the high-egg diet over a 12-month period produced no adverse effects on cardiovascular risk factors including triglycerides, total, LDL- and HDL-cholesterol, inflammatory markers, oxidative stress and glycaemia measurements [35].

Herron et al. [36] classified healthy people on the basis of their response to prolonged consumption of high dietary cholesterol. Hypo-responders, who experienced an increase in total cholesterol of < 0.05 mmol/L for each additional 100 mg of dietary cholesterol consumed, were 62.5%, whereas 37.5% experienced an increase in total cholesterol $\geq$0.41 mmol/L for each additional 100 mg and were considered hyper-responders. In the present study, 50% of the cohort exhibited a hyper-response to dietary cholesterol. Studies [36–38] indicated that dietary cholesterol intake from eggs significantly increases both serum LDL- and HDL-cholesterol, resulting in only a marginal change in the LDL-/HDL-cholesterol ratio. This is consistent with our findings. Indeed, the LDL-/HDL-cholesterol ratio did not change during the 3-month study and remained $\leq$ 2.01.

Historically, elevated LDL-cholesterol has been associated with an increased risk of CVD. However, non-HDL-cholesterol appears to be a better predictor for atherosclerotic vascular events [39]. Non-HDL-cholesterol is the total amount of lipoproteins containing apolipoprotein B (apoB), including very low-density lipoproteins and their metabolic remnants, intermediate density lipoproteins and chylomicrons. These lipoproteins can participate in atherogenesis by entering and getting trapped in the intima of the arterial wall [40,41]. The Multinational Cardiovascular Risk Consortium recently published an analytical tool to predict the long-term risk of CVD based on non-HDL-cholesterol levels. The reference value (hazard ratio, HR = 1.0) associated with the lowest risk of cardiovascular disease was set at 2.6 mmol/L or 100 mg/dL non-HDL-cholesterol in women and men. For individuals with the highest non-HDL-cholesterol levels ($\geq$220 mg/dL; HR= 1.9 in women and 2.3 in men), the CVD event rate over 30 years was predicted to be approximately three-to-four-times higher than in those with the lowest non-HDL-cholesterol (<100 mg/dL) [42]. According to this assessment model, the HR value between the start and the end of the present study was slightly increased (from 1.2 to 1.3) in both groups, suggesting that the increase in non-HDL-cholesterol seen during the study did not provide a worrisome additional risk on the incidence of CVD in the subjects.

Another indicator of CVD risk is ox-LDL, which are generally considered to be proatherogenic. Elevated ox-LDL levels are reported to be strongly positively linked to both atherosclerosis and inflammatory markers, including CRP, TNF-α and IL-6 [43,44]. In our study, the test group treated with ALA, DHA, RmA and PunA enriched eggs experienced a moderate but significant increase in plasma ox-LDL levels (from 51.89 to 63.06 U/L, $p < 0.05$). Currently, reference ranges for ox-LDL have not been established. Based on a prospective observational study of an apparently healthy and non-MetS population, a threshold of ox-LDL < 60 U/L has been defined for people at low risk of developing MetS. A range of 60 to 69 U/L would characterize people at relatively moderate risk, while a cut-off $\geq$ 70 U/L would indicate a high risk [45]. There was no change in inflammatory markers TNF-α and IL-6 in the test group, although a non-significant upward trend in hs-CRP was seen, suggesting the development of low-grade inflammation already observed in the control group since the start of the study.

In accordance with our results, previous studies have reported that relatively high levels of PUFA in the diet increased LDL particles susceptibility to lipid peroxidation [46–48]. The type and the amount of fat in the diet influence the fatty acid composition of lipoproteins and cell membranes, and may therefore affect the sensitivity of LDL and cells to oxidative damage. The more unsaturated the fatty acid, the more easily it oxidizes. Therefore, OA is less sensitive to oxidation compared with ALA, DHA, RmA and PunA [49]. If the latter are abundant in the LDL particles, they will promote the formation of ox-LDL. Yang et al. [50] comparing the oxidative stabilities of CLnA and CLA with their corresponding non-conjugated counterparts, ALA and linoleic acid, found that CLnA were the most unstable, followed by CLA, ALA and linoleic acid, in descending order. Unsurprisingly, DHA has also been found to enhance the susceptibility of cells to oxidative stress [46].

In addition, dietary fatty acids contribute to the pro-oxidant activity of arterial wall cells, since the fatty acid composition of the cell membrane influences cellular formation of reactive oxygen species (ROS) [48]. The increased production of ROS impairs nitric oxide (NO) bioavailability and prevents it from inducing vascular smooth muscle relaxation, which may lead to endothelial dysfunction. After a 3-month consumption of eggs enriched with mono- or poly-unsaturated fatty acids, erythrocyte HbNO levels were slightly reduced, indicating vascular oxidative stress and a reduction of NO bioavailability [51]. This is consistent with the weakly elevated hs-CRP (around 0.20 mg/dL) and the increase in ox-LDL seen in the two groups at month 3. High cholesterol levels have also been found to decrease the production of endothelial NO [52]. As the erythrocyte level of HbNO was shown to be a surrogate index of the endothelial NO production [53], this reduction of NO production was manifested by a decrease in HbNO levels, and was consistent with the observed cholesterol increase in both groups. However, the moderate decrease of the level of HbNO was not associated with impairment of the reactive hyperemia blood flow response assessed by peripheral arterial tonometry, likely due to the reduced number of subjects and the fact that peripheral arterial tonometry reflects more than an NO-dependent control.

5. Conclusions

This study, to our knowledge, is the first exploring the effects of eggs enriched with ALA, DHA, RmA and PunA on some metabolic parameters. The findings indicate that the consumption of these eggs during three months by subjects at high risk of MetS leads to a significant reduction in abdominal obesity, without improving other components of MetS including glycaemia-associated parameters. Although polyunsaturated fatty acids are expected to reduce the risk of CVD, they are prone to lipid peroxidation, and together with high cholesterol levels, may alter some markers of vascular oxidative stress. A limitation of this study is the small sample size, which may have prevented the emergence of certain differences between the two conditions of the study. Relevant outcomes (abdominal obesity, ox-LDL, HbNO), as well as their durability, deserve to be further studied on a larger sample and over a longer time frame. Another limitation is that to aid adherence, no strict dietary advice has been given regarding egg consumption. The participants did not change their eating behavior during the study period and the nutritional intake of cholesterol was not balanced. For future studies, prescribed diets or dietary guidance should be provided to participants in order to balance the cholesterol intake associated with egg consumption.

Author Contributions: Conceptualization, M.T.N.N., B.P. and Y.L.; methodology, M.T.N.N., B.P., I.L., C.G. and E.M.; validation, M.T.N.N., B.P., I.L., N.V.O., J.-L.B. and Y.L.; formal analysis, C.R.; investigation, M.T.N.N., B.P., I.L., N.V.O., L.D., E.V. and M.B.; resources, B.P., J.-L.B. and Y.L.; data curation, M.T.N.N.; writing—original draft preparation, M.T.N.N.; writing-reviewing and editing, B.P., I.L., N.V.O., C.R., J.-L.B. and Y.L.; visualization, M.T.N.N.; supervision, Y.L.; project administration, B.P. and Y.L.; funding acquisition, Y.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Ethical Committee of the Cliniques universitaires Saint-Luc (Brussels, Belgium) (Oil4Egg protocol approved on May 20, 2019), and conducted in accordance with the principles of the Declaration of Helsinki. The study is registered on ClinicalTrials.gov (identifier NCT04583657).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data sharing is not applicable to this article.

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