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Special Issue Reprint

Cardiometabolic Health in Relation to Diet and Physical Activity

Experimental and Clinical Evidence

Edited by
Abeer M. Mahmoud and Shane A. Phillips

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Cardiometabolic Health in Relation to Diet and Physical Activity: Experimental and Clinical Evidence

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Editors

Abeer M. Mahmoud
Shane A. Phillips

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Editors

Abeer M. Mahmoud
Department of Medicine,
Division of Endocrinology,
Diabetes, and Metabolism,
College of Medicine,
University of Illinois
at Chicago,
Chicago, IL, USA

Shane A. Phillips
Department of Physical
Therapy, College of Applied
Health Sciences, University of
Illinois at Chicago,
Chicago, IL, USA

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

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

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

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

ISBN 978-3-0365-8182-8 (Hbk)
ISBN 978-3-0365-8183-5 (PDF)

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About the Editors

Abeer M. Mahmoud

Abeer M. Mahmoud, MD, PhD is an Assistant Professor in the Department of Medicine, UIC. She received her MD degree and training as a Pathologist from Assiut University, Egypt. She served as a surgical pathologist at South Egypt Cancer Institute (2002–2008), before realizing her true passion was in biomedical research. She earned her Ph.D. in Pathology from the College of Medicine, UIC, in 2013, where she studied chemoprevention in prostate cancer. For her postdoctoral research (College of Applied Health Sciences, UIC, 2013–2017), she studied physiological and molecular outcomes of lifestyle interventions. She is funded by the National Institute of Health to study epigenetic modifications in morbidly obese patients and their impact on cardiometabolic functions. She also studies the role of adipose tissue secretions and exosomes in diabetes-associated vascular dysfunction and has conducted several exercise and nutrition trials to mitigate obesity and diabetes-associated co-morbidities.

Shane A. Phillips

Shane A. Phillips PT, PhD, FAHA is Professor and Associate Head in the Department of Physical Therapy at the University of Illinois at Chicago. Phillips has clinical expertise in physical therapy and cardiovascular rehabilitation. His PhD degree in physiology was completed at the Medical College of Wisconsin, and he has completed post-doctoral training in Cardiovascular Medicine. He is the Director of the Vascular Biology Laboratory in the College of Applied Health Sciences where he studies obesity, hypertension, and the control of blood flow and responses of microcirculation to surgery, diet and exercise interventions. Other research interests include the impact of cardiovascular risk factors such as high blood pressure, alcohol and high cholesterol on macro and microcirculatory function. His laboratory is funded by the National Institute of Health.

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Editorial

Editorial for the Special Issue "Cardiometabolic Health in Relation to Diet and Physical Activity: Experimental and Clinical Evidence"

Abeer M. Mahmoud [1,2]

[1] Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, College of Medicine, The University of Illinois at Chicago, Chicago, IL 60612, USA; amahmo4@uic.edu
[2] Department of Kinesiology and Nutrition, College of Applied Health Sciences, The University of Illinois at Chicago, Chicago, IL 60612, USA

This Special Issue seeks to compile a centered, influential, and well-referenced volume on the impact of diet and physical activity on various cardiometabolic risk factors. It is anticipated that the global prevalence of cardiometabolic illness, now a major health and economic burden, will continue to climb. There is mounting evidence that excessive caloric intake, poor food choices, and inactivity all contribute to the development of cardiometabolic risk factors such as altered glucose metabolism, insulin resistance, dyslipidemia, and hypertension [1]. This Special Issue aims to present robust data from recent experimental and observational research to establish the link between lifestyle variables such as diet and physical activity and cardiometabolic risk. This issue also includes studies that analyze the related molecular and subcellular pathways that mediate the link between these lifestyle factors and cardiometabolic risk.

Public health initiatives that promote healthy lifestyles, such as eating well and regularly exercising, are warranted in light of the persistently high prevalence of cardiometabolic risk factors. This issue features cutting-edge scientific assessments of food and lifestyle impacts on cardiometabolic health from eminent experts such as (1) the role of a healthy lifestyle in reducing stroke recurrence, (2) cultural preferences for healthy living and activities, (3) the impact of engaging in risky health behaviors in inducing cardiometabolic risk in Latinos, (4) the efficacy of web-based interventions in promoting a healthy lifestyle, (5) oral health as a predictive factor of eating preferences and hypertension in elderly, (6) the effectiveness of a combined citrulline and antioxidant supplements in improving endothelial function in postmenopausal women, (7) the acute effect of fiber-rich supplements in improving postprandial glucose and insulin levels, (8) fecal microbiota capsules in inducing bile acid metabolism and metabolic function, (9) the role of salt sensitivity genetic traits in high-fat diet-induced hypertension, and (10) the effectiveness of fasting interventions in preventing metabolic diseases.

Stroke continues to be the leading cause of disability and death worldwide, placing a significant strain on healthcare systems across the globe. Hypertension and oxidative stress play an essential role in the pathophysiology of stroke. In a cross-sectional study, Pham et al. [2]. investigated the moderating influence of antioxidative lifestyle factors in the link between hypertension and stroke recurrence. The authors of this study determined the ratio between the prooxidant capacity derived from smoking, drinking, being overweight, and obesity and the antioxidant capacity presented by a healthy diet that includes fruit and vegetable consumption and physical exercise in 951 stroke patients. In hypertensive individuals, a higher antioxidant/prooxidant ratio was associated with a decreased risk of stroke recurrence (odds ratio = 0.83; $p = 0.022$). This study supports the growing evidence that 75% of stroke cases can be prevented by adopting a healthy lifestyle [3,4].

It is important to note that some ethnic groups are more susceptible to cardiometabolic diseases than others. For instance, Latinxs are disproportionately affected by cardiometabolic

Citation: Mahmoud, A.M. Editorial for the Special Issue "Cardiometabolic Health in Relation to Diet and Physical Activity: Experimental and Clinical Evidence". *Nutrients* **2023**, *15*, 2903. https://doi.org/10.3390/nu15132903

Received: 4 June 2023
Accepted: 12 June 2023
Published: 27 June 2023

conditions. Therefore, it is imperative to focus on the culture-related behaviors and modifiable risk factors that place this population at high risk for cardiovascular and metabolic diseases. In a unique study by Sanchez-Johnsen et al. [5], the cultural preferences of the Latin population in Chicago were tested regarding healthy lifestyle factors such as diet and physical activity. Interviews with 203 Puerto Ricans and Mexicans demonstrated that the majority (60–80%) of the Latin population preferred interventions that are related to their culture, either the language used to communicate with them, exercise programs that include Latin or salsa movements or salsa music, dichos (Latino sayings), or cuentos (folktales or stories). The results suggest that therapies targeting one's way of life and perception of one's body image may reduce the risk of cardiometabolic illnesses.

Dr. Sanchez-Johnsen's research team also examined the role of engaging in multiple risky health behaviors in inducing cardiometabolic risk in Latinos. The team collected data on three health behaviors: (1) nutrition pattern, including vegetable and fruit intake; (2) physical activity; and (3) smoking. The study included 104 Puerto Rican and 99 Mexican men who reported health risk behaviors, 5% reported none, 30% reported one, 47% reported two, and 18% reported all three. These results emphasize the need to develop lifestyle interventions for Latinos that address various health risk behaviors associated with cardiometabolic diseases [6].

One of the lessons that were learned during the COVID-19 era is that remote interventions are needed that can access diverse populations, surmount limitations and barriers, and provide efficient approaches for promoting healthy behaviors. In their "Living Better" initiative, Mzquiz-Barberá et al. [7] implemented a self-administered web-based intervention to enhance lifestyle and assess the impact of this enhancement on long-term health benefits in obese patients with hypertension. The initiative also looked at the results of repeating the intervention during the COVID-19 epidemic (reintervention). All advantages gained in response to the "Living Better" program, such as reduced systolic and diastolic blood pressure, decreased body weight and body mass index, and improved dietary and physical activity behavior, were maintained throughout the duration of the study (3 years). These parameters also saw significant improvement in response to repeating this intervention during the COVID-19 pandemic.

In addition to nutrition and physical activity, oral health's contribution to overall health should not be underestimated. Oral health and tooth integrity significantly impact mastication ability, food selection, nutrition status, and general health, particularly in older adults. Marito et al. [8] examined the dietary preferences and patterns of 894 adults over 65 to comprehend the link between oral condition and hypertension in this geriatric population. Specifically, they sought to determine how these dietary preferences and patterns may explain the association between oral health and hypertension. Dietary intake was assessed using food frequency questionnaires, and oral health was evaluated by determining the number of teeth, the force of teeth closure, posterior occlusal support, mastication efficiency, oral hydration, and oral bacterial level. In addition to body mass index and age, inadequate posterior occlusion positively predicted hypertension (odds ratio = 1.72). The latter was also associated with a lack of fruit and vegetable consumption, indicating that oral health condition significantly impacts dietary preferences and, consequently, malnutrition-related illnesses such as hypertension.

Increasing research suggests that using dietary supplements may improve cardiometabolic health. Arginine was identified as one of the nutritional components that enhances vascular function; nevertheless, its oral administration is ineffective due to its degradation via arginase. Citrulline, unlike arginine, is not degraded by arginase and was reported to enhance plasma arginine and arterial elasticity and reduce resting blood pressure in middle-aged and elderly individuals [9]. Therefore, Figueroa et al. [10] tested the effect of four weeks of oral citrulline with and without the antioxidant glutathione on the vascular function of postmenopausal women, a population susceptible to endothelial dysfunction associated with deficiency of arginine due to oxidative stress. While citrulline administration increased arginine levels, only the combination of citrulline with the antioxidant

glutathione improved vasoreactivity of the brachial artery and attenuated the vasoconstriction in response to sympathoexcitation via the cold press test.

Incorporating more dietary fiber into one's diet has been shown to improve health and lower the likelihood of developing certain metabolic illnesses. Hammad Ullah et al. [11] measured the effect of a single meal rich in fibers on postprandial glucose and insulin levels. In this study, 40 normoglycemic participants with mild insulin resistance were randomized into two groups that received brewer's spent grain (BSG) extract-based food supplement or placebo with the provided breakfast meal. The BSG food supplement was rich in β-glucan, arabinoxylans, resistant starch, and other soluble fibers. After 120 min after the meal, the postprandial blood glucose and insulin values in the treatment group were considerably lower than the corresponding values in the placebo group. This study demonstrated the acute effect of a fiber-rich supplement in restoring postprandial blood glucose and insulin levels to those at baseline in individuals with modest insulin resistance. Further research is required to confirm that this acute effect is translated into long-term benefits, which will result in guidelines for supplementing the diets of individuals with mild insulin resistance with fiber.

Fecal microbiota is considered a novel direction in the field of supplementation, particularly concerning their effects on endogenous critical pathways such as bile acid metabolism. Bile acids are a family of bioactive metabolites whose function is mediated via bile acid receptors to promote metabolic health. The research conducted in the laboratory of Dr. Cummings suggests a therapeutic function for fecal microbiota transplantation in metabolic diseases by enhancing bile acid metabolism. In a double-blind, randomized study, obese patients treated with fecal microbiota capsules derived from a lean, healthy volunteer demonstrated improved gut bacterial bile acid metabolism and metabolic parameters. These findings were supplemented with analyses of stool specimens from respective patients to examine the microbial candidates that contribute the most to the metabolic improvement and the enhancement of gut bacterial bile acid metabolism. Several candidate bacteria that may contribute to the metabolic benefits of fecal microbiota transplantation and intestinal bacterial bile acid metabolism were identified in this research and stand as a valuable source for future research by the same group and others [12].

A relatively new area of study, nutrigenomics, focuses on the reciprocal relationship between what we eat and how our DNA interprets that food. In this special issue, original work by Lee et al. [13] showed that the same genetic traits that determine salt sensitivity may also influence the susceptibility to high-fat diet-induced hypertension. In this 12-week study, Dahl salt-resistant and salt-sensitive rats were administered either a high-fat diet (60% fat) or a chow diet (8% fat). Hypertension was observed in the salt-sensitive rodents but not the salt-resistant rats, even though both groups gained equal amounts of weight. In this research, genes implicated in adipogenesis, tissue remodeling, inflammation, and vascular function were analyzed in response to a high-fat diet in salt-sensitive and salt-resistant mouse models.

Finally, the current issue includes a comprehensive review article by Vrdoljak et al. [14] which summarizes the preclinical and clinical evidence regarding the impact of various forms of fasting on metabolic syndrome. Intermittent fasting and time-restricted feeding are two fasting interventions discussed in this article. This article discusses the inconsistent efficacy of these interventions in inducing tangible cardiometabolic benefits in humans relative to animal studies and the molecular and subcellular events that may have mediated the observed changes in the trials.

In conclusion, research that is now being conducted into the function of diet, exercise, and other lifestyle variables is continually attempting to shed light on the importance of these factors in sustaining cardiometabolic health. In addition, ongoing research is aiming to determine the molecular pathways that mediate the connection between one's lifestyle and current health state. In spite of this, there is a growing need for more clinical, translational, and mechanistic research to address outstanding issues about the discrepancy in results seen across various populations and between human and animal studies.

Funding: This research was funded by a grant from the National Institute of Health-NHLBI (Grant #R01 HL161386) (AMM).

Conflicts of Interest: The author declares no conflict of interest.

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Article

Association between Hypertension and Stroke Recurrence as Modified by Pro-oxidant–Antioxidant Balance: A Multi-Center Study

Thu T. M. Pham [1,2], Tuyen Van Duong [3,*], Lien T. K. Nguyen [4,5,6], Manh-Tan Vu [7,8], Khue M. Pham [2,9], Minh H. Nguyen [10], Thuc C. Luong [11,12], Binh N. Do [13,14], Lan T. H. Le [15,16,17], Nga H. Dang [15,18], Thao T. P. Nguyen [19], Hoang P. Le [20], Cuong Q. Tran [21,22], Kien T. Nguyen [23], Chaur-Jong Hu [24,25], Chang-Chuan Chan [26,27,28], Hui-Chuan Hsu [1] and Chyi-Huey Bai [1,*]

1 School of Public Health, College of Public Health, Taipei Medical University, Taipei 110-31, Taiwan; phamminhthu.ytcc@gmail.com (T.T.M.P.); gingerhsu@tmu.edu.tw (H.-C.H.)
2 Faculty of Public Health, Hai Phong University of Medicine and Pharmacy, Hai Phong 042-12, Vietnam; pmkhue@hpmu.edu.vn
3 School of Nutrition and Health Sciences, Taipei Medical University, Taipei 110-31, Taiwan
4 Rehabilitation Department, Hanoi Medical University, Hanoi 115-20, Vietnam; lienrehab@hmu.edu.vn
5 Rehabilitation Center, Bach Mai Hospital, Hanoi 115-19, Vietnam
6 Rehabilitation Department, Viet Duc University Hospital, Hanoi 110-17, Vietnam
7 Department of Internal Medicine, Haiphong University of Medicine and Pharmacy, Hai Phong 042-12, Vietnam; vmtan@hpmu.edu.vn
8 Cardiovascular Department, Viet Tiep Friendship Hospital, Hai Phong 047-08, Vietnam
9 President Office, Hai Phong University of Medicine and Pharmacy, Hai Phong 042-12, Vietnam
10 School of Preventive Medicine and Public Health, Hanoi Medical University, Hanoi 121-08, Vietnam; drminh.ttytlc@gmail.com
11 Director Office, Military Hospital 103, Hanoi 121-08, Vietnam; lcthuc@gmail.com
12 Department of Cardiology, Cardiovascular Center, Military Hospital 103, Hanoi 121-08, Vietnam
13 Department of Infectious Diseases, Vietnam Military Medical University, Hanoi 121-08, Vietnam; nhubinh.do@vmmu.edu.vn
14 Division of Military Science, Military Hospital 103, Hanoi 121-08, Vietnam
15 Training and Direction of Healthcare Activity Center, Thai Nguyen National Hospital, Thai Nguyen City 241-24, Vietnam; lanhuong.bvtutn@gmail.com (L.T.H.L.); danghoangngatn@gmail.com (N.H.D.)
16 Biochemistry Department, Thai Nguyen National Hospital, Thai Nguyen City 241-24, Vietnam
17 Director Office, Thai Nguyen National Hospital, Thai Nguyen City 241-24, Vietnam
18 Department of Quality Control, Thai Nguyen National Hospital, Thai Nguyen City 241-24, Vietnam
19 Institute for Community Health Research, University of Medicine and Pharmacy, Hue University, Hue 491-20, Vietnam; nguyenthiphuongthao@hueuni.edu.vn
20 Department of Internal Medicine, University of Medicine and Pharmacy, Hue University, Hue 491-20, Vietnam; lephuochoang@hueuni.edu.vn
21 Director Office, Thu Duc City Health Center, Ho Chi Minh City 713-10, Vietnam; quoccuong.mph@gmail.com
22 Faculty of Health, Mekong University, Vinh Long 852-16, Vietnam
23 Department of Health Promotion, Faculty of Social and Behavioral Sciences, Hanoi University of Public Health, Hanoi 119-10, Vietnam; ntk1@huph.edu.vn
24 Department of Neurology, School of Medicine, College of Medicine, Taipei Medical University, Taipei 110-31, Taiwan; chaurjongh@tmu.edu.tw
25 Department of Neurology, Taipei Medical University Shuang Ho Hospital, New Taipei City 235-61, Taiwan
26 Institute of Environmental and Occupational Health Sciences, College of Public Health, National Taiwan University, Taipei 100-55, Taiwan; ccchan8082@gmail.com
27 Innovation and Policy Center for Population Health and Sustainable Environment (Population Health Research Center, PHRC), College of Public Health, National Taiwan University, Taipei 100-55, Taiwan
28 Global Health Program, College of Public Health, National Taiwan University, Taipei 100-55, Taiwan
* Correspondence: duongtuyenvna@gmail.com (T.V.D.); baich@tmu.edu.tw (C.-H.B.)

Citation: Pham, T.T.M.; Duong, T.V.; Nguyen, L.T.K.; Vu, M.-T.; Pham, K.M.; Nguyen, M.H.; Luong, T.C.; Do, B.N.; Le, L.T.H.; Dang, N.H.; et al. Association between Hypertension and Stroke Recurrence as Modified by Pro-oxidant–Antioxidant Balance: A Multi-Center Study. *Nutrients* **2023**, *15*, 2305. https://doi.org/10.3390/nu15102305

Academic Editors: Shane Phillips and Abeer M. Mahmoud

Received: 11 April 2023
Revised: 10 May 2023
Accepted: 12 May 2023
Published: 14 May 2023

Abstract: Background: Hypertension and oxidative stress are involved in the pathophysiological mechanism of stroke. We aimed to investigate the modification impact of the pro-oxidant–antioxidant balance (PAB) on the association between hypertension and stroke recurrence (SR). Methods: A cross-sectional design was conducted from December 2019 to December 2020 in 951 stroke patients

in six hospitals across Vietnam. Hypertension was defined using antihypertensive medication or systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg. PAB was estimated using weighting methods based on smoking, drinking, and overweight/obesity with pro-oxidant capacity, diet quality, fruit intake, vegetable intake, and physical activity with antioxidant capacity. The higher PAB scores indicated a beneficial balance shifting toward antioxidant dominance. SR was diagnosed by neurologists. Moreover, sociodemographic and health conditions were included as covariates. Multiple logistic regression analyses were used to explore the associations and interactions. Results: The hypertension and SR proportions were 72.8% and 17.5%, respectively. hypertension was associated with an increased SR likelihood (odds ratio (OR) = 1.93; $p = 0.004$), whereas a higher PAB score was associated with a lowered SR likelihood (OR = 0.87; $p = 0.003$). Moreover, hypertension interacting with every one-point increment of PAB was associated with a lowered SR likelihood (OR = 0.83; $p = 0.022$). Conclusions: The harmful impact of hypertension on SR could be alleviated by PAB. The interplay of health behaviors should be highlighted in the intervention strategies for stroke prevention.

Keywords: stroke recurrence; hypertension; oxidative stress; pro-oxidant–antioxidant balance; health behavior

1. Introduction

To date, stroke remains the most common cause of disability and mortality world-wide, which leads to a substantial global health burden [1]. The highest rate of stroke incidence was found in Southeast Asian countries, including Vietnam [2]. Although multilevel and multisectoral prevention strategies have been applied, the stroke recurrence (SR) rate has not changed over the years [3,4]. Moreover, the risk of SR increases over time [5], causing continuously increasing issues in societies and medical systems around the world [6], as well as in Vietnam [7]. Therefore, closing the gap in stroke prevention strategies is a high priority.

About 90% of strokes are caused by the presence of modifiable risk factors, and the regulation of core health behaviors (such as diet, physical activity, weight, and smoking) could avert about 75% of this burden [6,8]. Hypertension (HTN) is the most frequent danger for stroke and recurrent events [9,10]. Although hypertensive management is the most important primary and secondary prevention strategy for stroke, managing blood pressure in stroke patients is complex and challenging [11]. Moreover, evidence-based prevention strategies are not obviously improving modifiable risk factors and recurrent cardiovascular events (including stroke) [12]. Therefore, exploring factors that could be accessible for modifying the harmful impact of HTN on SR is necessary for prevention strategies.

Oxidative stress (OS) is characterized by the pro-oxidant–antioxidant balance (PAB) shifting toward the pro-oxidant predominance, resulting in molecular damage [13–15]. The existing evidence showed that the OS response could derive from endogenous (e.g., metabolic processes) and exogenous sources (e.g., core health behaviors), which are involved in the pathological mechanism of chronic diseases [16], including stroke [17–19]. In the endogenous aspect, OS is linked closely to chronic inflammatory disorders, which were identified as the major triggers for cardiovascular diseases (CVDs). The close interaction between OS and inflammatory response was reported regarding the overexpression of reactive oxygen species (ROS)-producing enzymes (e.g., NADPH NOXs) or aggravated inflammatory phenotypes in the absence of antioxidant defense proteins (e.g., glutathione peroxidases, heme oxygenase-1, and superoxide dismutase) [20]. Moreover, OS plays a critical role in the mechanism of CVDs through endothelial dysfunction, of which oxidative enzyme systems (e.g., xanthine oxidase and NADPH oxidase) contribute to the inactivation of nitric oxide—an endothelial regulator, leading to endothelial dysfunction [21]. In the exogenous aspect, potential diets and nutraceuticals could reduce OS and display antioxidant effects,

which represents a therapeutic target in CVDs [21]. Additionally, regulating PAB could be a promising strategy for stroke therapeutics [22].

The PAB can be approached from pro-oxidative and antioxidative perspectives, and both can be considered in terms of their individual chemical constituents or their pooled effect [23]. Pro-oxidants can be from intracellular sources (such as NADPH NOXs, peroxisomes, and mitochondria) and external sources (such as pollutants, ultraviolet light, and ionizing) [24], whereas endogenous antioxidants involve the products of cellular metabolism (e.g., peroxiredoxin, catalase, and superoxide dismutase), and exogenous antioxidants involve diets and medications [24]. In the literature, the PAB has been directly estimated in saliva, urine, plasma, and serum [23], demonstrating the contribution of exogenous and endogenous agents. However, for example, serum PAB cannot reflect each agent's specific pro-oxidant and antioxidant potentials and the complex interaction between the agents. Therefore, an indirect measurement tool based on lifestyle, nutrition, and medication factors was developed and validated to evaluate the individual PAB status and its impact on health outcomes [25–27]. However, the evidence regarding the indirect estimation of PAB based on health behaviors impacting SR is limited. Therefore, we aimed to examine the relationship between health behavior-based PAB and SR. Further, the modification role of PAB in the association between HTN and SR was explored.

2. Materials and Methods

2.1. Study Population

A cross-sectional study was launched from December 2019 to December 2020 to enroll patients with stroke in Vietnam. Due to the COVID-19 crisis, six invited hospitals agreed to participate in our study. Stroke patients were identified by neurologists using the 10th revision of the International Classification of Disease (ICD-10) coding I60–I69 [28], who were recruited from rehabilitation, neurology, and cardiovascular departments. Patients with stable stroke conditions (e.g., a Mini-Mental State Examination score of $\geq$22, vital signs within normal limits, recovery after the acute stroke and hospitalized at least one month), aged $\geq$ 18 years, and being able to reply to questions were eligible for selection. Moreover, we excluded patients with aphasia and impairment of vision and cognition. A minimum sample of 715 was estimated using SAS v9.4 software based on eight predictors in multiple logistic regression with a power of 0.8 and an alpha of 0.1. A satisfactory sample of 951 qualified patients was recruited for the final analysis. This study was reviewed and approved by the Institutional Ethical Review Committee of the Hanoi School of Public Health, Vietnam (IRB No. 498/2019/YTCC-HD3 and No. 312/2020/YTCC-HD3).

2.2. Data Collection and Measurement

2.2.1. Data Collection Procedure

A four-hour training session regarding data collection and infection control (e.g., wearing a mask, washing hands, and physical distancing) during the COVID-19 pandemic was provided for the interviewers (including medical students, doctors, and nurses) according to the guidelines provided by the World Health Organization (WHO) [29] and the Ministry of Health in Vietnam [30]. Then, patients were asked to sign an informed consent form before participating in a 30 min face-to-face survey at the bedside of each patient.

2.2.2. Assessment of Outcome

Stroke recurrence (SR) was identified by asking patients and their families whether the current hospitalization was the first or recurrent (including the number of times) stroke. Then, patients were regrouped into the first stroke vs. recurrent stroke to facilitate the analysis because of the limited sample size for the number of times of recurrent events.

2.2.3. Assessment of Hypertension

A standard clinical manual aneroid sphygmomanometer was used for three-time measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP). The

average SBP and DBP were computed. Additionally, participants' information about taking antihypertensive medication was obtained. Hypertension was identified as using any antihypertensive medication, SBP $\geq$ 140 mmHg, or DBP $\geq$ 90 mmHg [31,32].

2.2.4. Assessment of Pro-oxidant–Antioxidant Balance

The PAB was indirectly established in the literature based on multiple components and comparable weighting methods (such as equal-weighted, study data-based, literature review-derived, and Bayesian methods) [33,34]. In the current study, the PAB components were defined based on core health behaviors related to stroke, including overweight/obesity, smoking, and drinking with pro-oxidant properties, physical activity, diet quality, fruit intake, and vegetable intake with antioxidant properties.

- Drinking frequency and smoking status were self-reported. Body mass index (BMI, kg/m^2) was computed and classified into under/normal weight, overweight, and obesity [35,36].
- Diet quality was assessed using the Dietary Approaches to Stop Hypertension Quality (DASH-Q) questionnaire [37], and the 10-item DASH-Q questionnaire was validated for use in the Vietnamese context [38]. Patients were asked how many days they ate the food items over the previous seven days. A DASH-Q score was summed (ranging between 0 and 70) and categorized into tertiles, with greater tertiles indicating healthier diet quality.
- Fruit intake and vegetable intake were assessed using the STEPwise questionnaire with four typical questions that patients were asked about their consumption of fruit and vegetables in terms of the number of days per week and the number of servings per day. The WHO STEPwise questionnaire was validated in the Vietnamese context [39]. According to WHO recommendations adopted in Asian countries (including Vietnam) [40], a minimum daily intake of five servings of fruit and vegetables (including two fruit and three vegetable servings) was proposed to prevent chronic diseases.
- Physical activity was assessed using a short version of the International Physical Activity Questionnaire (IPAQ) [41], which was validated for use in the Vietnamese context [42,43]. Patients were asked how many minutes per day and days per week over the previous seven days they undertook sitting, walking, and moderate and vigorous activities. Then, the sum score of minutes per week for each of those four activities was multiplied by 1, 3.3, 4, and 8, respectively, to estimate the equivalent metabolic task scored in minutes per week (MET/min-wk) [44]. A higher MET score indicated a more intensive PA level. According to WHO [45] and the Physical Activity Guidelines Advisory Committee [46], a minimum PA level of 600 to 1200 MET/min-wk is recommended for adults with chronic conditions or disabilities.

All components were categorized into three levels and assigned 0, 1, or 2 values, accordingly. Then, the PAB score was calculated by summing all components after weighting by multiplying the values with -1 for pro-oxidant and +1 for antioxidant (Table 1). The PAB score ranged from -6 to 8, with higher PAB scores indicating a beneficial balance shifting toward antioxidant dominance.

2.2.5. Assessment of Covariates

Socio-demographic factors (including age, gender, educational attainment, occupation, social status, marital status, and ability to pay for medication) were self-reported.

Stroke classification was categorized based on the ICD-10 into infraction (including cerebral infarction (I63)), hemorrhage stroke (including subarachnoid hemorrhage (I60), intracerebral hemorrhage (I61), other nontraumatic intracranial hemorrhage (I62)), and others (including stroke not specified as hemorrhage or infarction (I64); occlusion and stenosis of precerebral arteries, not resulting in cerebral infarction (I65); occlusion and stenosis of cerebral arteries, not resulting in cerebral infarction (I66); other cerebrovascular diseases (I67); cerebrovascular disorders in diseases classified elsewhere (I68); and sequelae of cerebrovascular disease (I69)).

Table 1. Categories and weights of pro-oxidant–antioxidant balance components.

Component	Categories	Definition	Weights
Pro-oxidants			
Smoking	Never	Never smoked	0
	Used to	Former smoker	−1
	Current	Currently smokes	−2
Drinking	None	Never drink	0
	Moderate	Drink 1–4 times/month on average	−1
	Heavy	Drink daily or at least ≥ 3 times/week	−2
BMI (kg/m^2)	Under/normal weight	BMI < 23	0
	Overweight/obesity	23 ≤ BMI < 27.5	−1
	Obesity	BMI ≥ 27.5	−2
Anti-oxidants			
Fruit intake	Low	<2 servings/day	0
	Moderate	2 servings/day	+1
	High	>2 servings/day	+2
Vegetable intake	Low	<3 servings/day	0
	Moderate	3 servings/day	+1
	High	>3 servings/day	+2
Diet quality	Low	DASH-Q ≤ 24	0
	Moderate	24 < DASH-Q ≤ 35	+1
	High	DASH-Q > 35	+2
Physical activity (MET-min/wk)	Low	MET < 600	0
	Moderate	600 ≤ MET ≤ 1200	+1
	High	MET > 1200	+2

Comorbidity was judged using the 16-item Charlson comorbidity index (CCI), which is validated and used widely in Vietnam [47,48]. In the current study, items of cerebrovascular disease or stroke and dementia were not included in the comorbid conditions due to the study participants and exclusion criteria, respectively. Additionally, the item of depression was not counted as a comorbidity to avoid the duplicated assessment. The remaining items were regrouped into none vs. one/more CCI to simplify the analysis.

Depressive symptoms were assessed using a two-item patient health questionnaire (PHQ-2), which was suggested for use in busy medical settings [49,50]. Patients were asked about the frequency of being affected by positive or depressed moods in the past two weeks and were rated on a three-point scale from 0 (not at all) to 3 (nearly every day). The PHQ-2 score ranged from 0 to 6, and a score ≥ 2 was suggested for clinicians to ensure that the few cases of depression were not overlooked [51].

Health literacy (HL) (including three domains of healthcare, disease prevention, and health promotion) was estimated using a 12-item short-form survey (HLS-SF12). The difficulty of performing each item was rated from 1 (very difficult) to 4 (very easy) based on a 4-point Likert scale. The HL index (the standardized HL indices) was calculated using the following formula:

$$Index = \frac{(mean - 1) \times 50}{3}$$

in which *mean* displays the average of 12 items, while *1*, *3*, and *50* display the minimal possibility of the mean, the range of the mean, and the chosen maximum HL index score, respectively. The HL index varied from 0 (worse HL) to 50 (best HL).

2.3. Statistical Analysis

First, descriptive analyses were performed, and the independent sample T-test (or Kruskal–Wallis test) and chi-square test were used for continuous and categorical variables,

respectively. Second, logistic regression analysis was used to examine the relationship between HTN and PAB with SR. The independent variables (IVs) with $p < 0.2$ in the bivariate analysis were selected for the multiple regression models. The multicollinearity among those IVs was controlled by examining their correlation using Spearman's correlation. Because moderate correlations were found between age and occupation (*rho* = 0.34) and between gender and PAB (*rho* = −0.35) (Table S1), the representative variables were selected for multiple analysis models, including age, education, ability to pay for medication, stroke classification, depressive symptoms, and CCI. Third, interaction analysis was used to explore the combined impact of HTN and PAB on SR. Furthermore, the results of the interaction model were visualized via a simple slope analysis using PROCESS Macro of SPSS for moderation analysis. The slope plots were drawn using the estimated values of SR probability for two categories of HTN (yes vs. no) by three levels of PAB (one standard deviation below the mean (−1SD), the mean, and one standard deviation above the mean (+1SD)). All analyses were conducted using SPSS version 22 (IBM Corp., Armonk, NY, USA), and the *p*-value < 0.05 was defined as a statistically significant result.

3. Results

3.1. Characteristics of Stroke Patients

Out of 951 patients with stroke, the proportions of SR and HTN were 17.5% and 72.8%, respectively. The age ranged from 19 to 99 years, and the proportion of patients aged ≥65 was 53.7%. Compared to participants with the first stroke, those with SR had a higher percentage of age ≥ 65 ($p = 0.011$), were men ($p = 0.042$), were retired or infirm ($p = 0.021$), and had an infarction stroke ($p = 0.012$), HTN ($p < 0.001$), and CCI ($p < 0.001$) (Table 2).

Table 2. Characteristics and stroke recurrence (*n* = 951).

Variables	Total	First Stroke	Recurrent Stroke	
	***n* (%)**	**(*n* = 785, 82.5%)**	**(*n* = 166, 17.5%)**	***p***
Socio-demographics				
Age (years)				0.011
<65	440 (46.3)	378 (48.2)	62 (37.3)	
≥65	511 (53.7)	407 (51.8)	104 (62.7)	
Gender				0.042
Woman	388 (40.8)	332 (42.3)	56 (33.7)	
Man	563 (59.2)	453 (57.7)	110 (66.3)	
Occupation				0.021
Working	518 (54.5)	441 (56.2)	77 (46.4)	
Retirement or infirmity	433 (45.5)	344 (43.8)	89 (53.6)	
Education attainment				0.363
Illiterate or elementary	216 (22.7)	181 (23.1)	34 (20.5)	
Junior high school	257 (27.1)	204 (26.0)	53 (31.9)	
Senior high school	251 (26.4)	206 (26.3)	45 (27.1)	
College/university or higher	227 (23.9)	193 (24.6)	34 (20.5)	
Ability to pay for medication				0.129
Very or fairly difficult	423 (44.5)	358 (45.6)	65 (39.2)	
Very or fairly easy	528 (55.5)	427 (54.4)	101 (60.8)	
Marital status				0.979
Married	837 (88.0)	691 (88.0)	146 (88.0)	
Single or Widowed/Divorced/Separated	114 (12.0)	94 (12.0)	20 (12.0)	
Social status				0.335
Low	111 (11.7)	88 (11.2)	23 (13.9)	
Middle or high	840 (88.3)	697 (88.8)	143 (86.1)	
HL index (mean ± SD)	23.4 ± 10.0	23.5 ± 10.0	22.8 ± 9.9	0.426

Table 2. *Cont.*

Variables	Total	First Stroke	Recurrent Stroke	
	***n* (%)**	**(*n* = 785, 82.5%)**	**(*n* = 166, 17.5%)**	***p***
Health conditions				
Stroke classification				0.012
Hemorrhage	220 (23.2)	196 (25.0)	24 (14.5)	
Infraction	637 (67.1)	511 (65.3)	126 (75.9)	
Others	92 (9.7)	76 (9.7)	16 (9.6)	
Depressive symptoms				0.145
No	461 (48.5)	372 (47.4)	89 (53.6)	
Yes	490 (51.5)	413 (52.6)	77 (46.4)	
CCI				<0.001
No	476 (50.1)	415 (52.9)	61 (36.7)	
Yes	475 (49.9)	370 (47.1)	105 (63.3)	
Hypertension				<0.001
No	259 (27.2)	232 (29.6)	27 (16.3)	
Yes	692 (72.8)	553 (70.4)	139 (83.7)	
PAB (median, IQR)	1.0 (0.0, 3.0)	1.0 (0.0, 3.0)	0.0 (−1.0, 2.0)	<0.001

Abbreviation: HL, health literacy; SD, standard deviation; CCI, Charlson comorbidity index; PAB, pro-oxidant–antioxidant balance; IQR, interquartile range.

3.2. Determinants of Stroke Recurrence

Among the investigated factors, the multiple analysis results (Table 3) showed that HTN (odds ratio, OR, 1.93; 95% confidence interval, 95%CI, 1.23, 3.04; $p = 0.004$) and CCI (OR, 1.55; 95%CI; 1.08, 2.23; $p = 0.017$) were associated with a higher likelihood of SR. Whereas PAB was associated with a lower likelihood of SR (OR, 0.87; 95%CI, 0.80, 0.95; $p = 0.003$). Moreover, hemorrhage stroke had a lower likelihood of recurrence compared to infarction stroke (OR, 0.55; 95%CI, 0.34, 0.90; $p = 0.019$).

Table 3. Determinants of stroke recurrence ($n = 951$).

Variables	Stroke Recurrence					
	Crude Model			Adjusted Model		
	OR *	**95%CI ***	***p* ***	**OR ****	**95%CI ****	***p* ****
Socio-demographics						
Age (years)						
<65	1.00			1.00		
≥65	1.55	1.10–2.19	0.012	1.28	0.88–1.85	0.191
Gender						
Woman	1.00					
Man	1.44	1.01–2.04	0.042			
Occupation						
Working	1.00					
Retirement or infirmity	1.48	1.05–2.07	0.022			
Education attainment						
Illiterate or elementary	1.00			1.00		
Junior high school	1.38	0.86–2.22	0.181	1.22	0.74–2.01	0.420
Senior high school	1.16	0.71–1.89	0.544	1.17	0.70–1.95	0.546
College/university or higher	0.93	0.55–1.57	0.808	0.87	0.50–1.52	0.638
Ability to pay for medication						
Very or fairly difficult	1.00			1.00		
Very or fairly easy	1.30	0.92–1.83	0.129	1.18	0.82–1.71	0.361
Marital status						
Married	1.00					
Single or Widowed/Divorced/Separated	1.01	0.60–1.68	0.979			

Table 3. *Cont.*

Variables	Stroke Recurrence					
	Crude Model			Adjusted Model		
	OR *	95%CI *	*p* *	OR **	95%CI **	*p* **
Social status						
Low	1.00					
Middle or high	0.78	0.47–1.28	0.336			
HL index	0.99	0.97–1.01	0.426			
Health conditions						
Stroke classification						
Hemorrhage	1.00			1.00		
Infraction	2.01	1.26–3.21	0.003	1.79	1.10–2.91	0.019
Others	1.71	0.86–3.41	0.121	1.68	0.82–3.43	0.152
Depressive symptoms						
No	1.00			1.00		
Yes	0.77	0.55–1.09	0.145	0.79	0.56–1.12	0.194
CCI						
No	1.00			1.00		
Yes	1.93	1.36–2.72	<0.001	1.55	1.08–2.23	0.017
Hypertension						
No	1.00			1.00		
Yes	2.16	1.39–3.35	0.001	1.93	1.23–3.04	0.004
PAB	0.84	0.77–0.91	<0.001	0.87	0.80–0.95	0.003

Abbreviation: OR, odds ratio; CI, confidence interval; HL, health literacy; CCI, Charlson comorbidity index; PAB, pro-oxidant–antioxidant balance. * Results of bivariate logistic regression analysis. ** Results of multivariate logistic regression analysis adjusted for age, education, ability to pay for medication, stroke classification, depressive symptoms, and CCI.

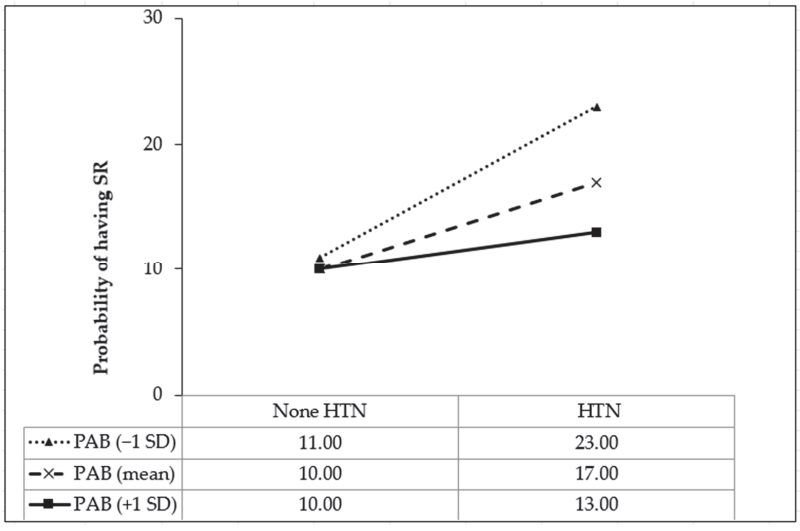
3.3. Modification Impact of PAB on the Association between HTN and Stroke Recurrence

As shown in Table 4, compared to stroke patients without HTN and with the lowest PAB scores, those with HTN had an increased likelihood of SR (OR, 2.06; 95%CI, 1.28, 3.32; $p = 0.003$). However, the likelihood of SR decreased in stroke patients with HTN and with every one-point increment of PAB (OR, 0.83; 95%CI, 0.66, 0.94; $p = 0.022$). Moreover, the results of interaction were visualized in Figure 1. Simple slope analysis showed that the negative impact of HTN on SR was lowered by higher PAB values from −1SD (OR, 2.43; 95%CI, 1.29. 4.57; $p = 0.005$) to the mean (OR, 1.82; 95%CI, 1.16, 2.88; $p = 0.008$) and +1SD (OR, 1.36; 95%CI, 1.13, 2.58; $p = 0.003$).

Table 4. Interaction of hypertension and pro-oxidant–antioxidant balance on stroke recurrence ($n = 951$).

Interaction	Stroke Recurrence					
	Crude Model			Adjusted Model		
	OR *	95%CI *	*p* *	OR **	95%CI **	*p* **
Non-HTN × PAB (lowest score)	1.00			1.00		
HTN × PAB (lowest score)	2.12	1.33–3.38	0.002	2.06	1.28–3.32	0.003
Non-HTN × PAB (1-point increment)	0.95	0.78–1.16	0.677	0.90	0.82–1.23	0.934
HTN × PAB (1-point increment)	0.86	0.69–0.97	0.012	0.83	0.66–0.94	0.022

Abbreviation: OR, odds ratio; CI, confidence interval; HTN, hypertension; PAB, pro-oxidant–antioxidant balance. * Results of bivariate logistic regression analysis. ** Results of multivariate logistic regression analysis, adjusted for age, education, ability to pay for medication, stroke classification, depressive symptoms, and comorbid conditions.



	None HTN	HTN
PAB (−1 SD)	11.00	23.00
PAB (mean)	10.00	17.00
PAB (+1 SD)	10.00	13.00

Figure 1. Simple slope plot for the interaction between hypertension and pro-oxidant–antioxidant balance on stroke recurrence (n = 951). HTN, hypertension; PAB, pro-oxidant–antioxidant balance; SD, standard deviation.

4. Discussion

The current findings emphasize the positive impact of PAB on lowering SR, especially regarding the moderating role of PAB in mitigating the negative impact of HNT on SR.

Although OS is involved in the pathological mechanism of stroke, the study of OS in stroke patients is challenging to undertake because of the complexly interrelated processes. Further, OS is caused by numerous exogenous factors, and the combined (either addictive or synergistic) influences of those factors on the OS process should be considered. Therefore, a global estimation of the PAB score was established based on various dietary, lifestyle, and medication components [25]. In the literature, a low PAB score (reflecting excessive OS) was associated with poor health outcomes (such as chronic kidney diseases and all-cause mortality) [25], but little is known about PAB score and SR. Previous studies directly estimated the plasma PAB concentration and found heterogeneity associations between PAB and SR. Although the redox unbalances were greater in patients within one month of stroke onset than in their controls [52], the serum PAB concentration at the acute stroke phase was not associated with SR within five years [53]. To our knowledge, the current study was the first to find that a higher health behavior-based PAB score reflecting antioxidant properties could reduce the likelihood of SR.

The relationship between OS and inflammation is a vicious circle that links to HTN [54]. In stroke patients, persistent inflammatory responses are activated in those with HTN, which leads to an additional chain of OS procedures, and the HTN-OS correlation contributes to increasing SR [55]. That is why eliminating the free radical agents in the OS processes via "scavengers" is able to decrease the adverse consequences of stroke [55]. Previous studies showed that although the association between the antioxidative and anti-inflammatory capacities of a single dietary component with the hypotensive impact was not clear, adding such a component may become a new method for controlling the balance in OS–inflammation crosstalk [56]. Our findings supported the rationale that a greater PAB score could minimize the deleterious influence of HTN on SR probability. In the current study, the PAB score was estimated based on diet quality, fruit and vegetable intake, and physical activity in regard to anti-oxidative capacity. Therefore, the interplay of those components should be taken into consideration for the balanced regulation of OS and inflammation, as well as for preventing SR.

In recent decades, dietary nutrients have been used a novel intervention for building cardiovascular and neurological health due to their antioxidant properties [57]. However, the mechanism regarding the relationship between diet nutrients and neuro-cardiological diseases is not yet well defined, that metabolic control (including lipid, ROS, and energy metabolisms), immunological regulation (including astrocyte and microglial activation), and epigenetic modification (including non-coding RNA, DNA methylation, and histone modification) were assumed to be involved. Regarding dietary nutrients, the intervention programs focused on two forms of diet, including dietary supplements (such as vitamins and plant extracts) and dietary restriction (such as the DASH diet with decreasing cholesterol, total fat, and saturated fat and increasing fibers and minerals). Moreover, fruits and vegetables are rich sources of vitamins and plant extracts containing polyphenolic components, which are potent antioxidants and are reported to be beneficial to cardiovascular health [58]. However, the excessive intake of antioxidative dietary nutrients (e.g., iron and vitamin A) could lead to the side effects, such as cell death, because of ROS overproduction, causing OS. Additionally, there are anti-nutrient compounds contained in fruits and vegetables (such as tannins, oxalates, and lectins), which may threaten health [59]. Therefore, the approach of PAB needs to consider both the individual pro-oxidant and antioxidant components and their mutual effects.

The present study is the first to assess the potential role of PAB score in altering the relationship between HTN and SR. However, several limitations should be noted. First, the associations were recognized, but the causal inference could not be established in a cross-sectional study. Second, as the study participants were recruited in stable conditions of stroke, the findings could apply only to mild and moderate stroke patients but not to those with severe stroke conditions. However, the sample size was satisfactory for representativeness, with a power of evidence of up to 90%. Third, the questionnaires were responded to via self-reporting, and the "standard serving" size of food was not defined, which may lead to underestimation.

5. Conclusions

The current findings highlighted the critical contribution of PAB to prevent SR and mitigate the negative impact of hypertension on SR. Because multiple factors contribute to oxidative stress, we suggest that health promotion programs should include multiple dimensions to promote an overall healthy lifestyle to reduce oxidative stress. In the future research, the interplay of health behaviors should be considered in the strategic intervention of balancing the oxidative stress–inflammation relationship and preventing SR.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu15102305/s1, Table S1: Spearman's correlation (*rho*) among the studied variables (n = 951).

Author Contributions: Conceptualization, T.T.M.P., L.T.K.N., M.-T.V., K.M.P., M.H.N., T.C.L., B.N.D., L.T.H.L., N.H.D., T.T.P.N., H.P.L., C.Q.T., K.T.N., C.-J.H., C.-C.C., H.-C.H., C.-H.B. and T.V.D.; methodology, T.T.M.P., L.T.K.N., M.-T.V., K.M.P., M.H.N., T.C.L., B.N.D., L.T.H.L., N.H.D., T.T.P.N., H.P.L., C.Q.T., K.T.N., C.-J.H., C.-C.C., H.-C.H., C.-H.B. and T.V.D.; software, T.T.M.P., C.-H.B. and T.V.D.; formal analysis, T.T.M.P., C.-H.B. and T.V.D.; investigation, T.T.M.P., L.T.K.N., M.-T.V., K.M.P., M.H.N., T.C.L., B.N.D., L.T.H.L., N.H.D., T.T.P.N., H.P.L., C.Q.T., K.T.N. and T.V.D.; resources, C.-H.B. and T.V.D.; writing—original draft preparation, T.T.M.P., C.-H.B. and T.V.D.; writing—review and editing, T.T.M.P., L.T.K.N., M.-T.V., K.M.P., M.H.N., T.C.L., B.N.D., L.T.H.L., N.H.D., T.T.P.N., H.P.L., C.Q.T., K.T.N., C.-J.H., C.-C.C., H.-C.H., C.-H.B. and T.V.D.; supervision, C.-H.B. and T.V.D.; project administration, T.T.M.P., T.T.P.N., C.-H.B. and T.V.D.; funding acquisition, C.-H.B. and T.V.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science and Technology Council, Taiwan (grant number: MOST 111-2410-H-038-008-MY2).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethical Review Committee of Hanoi School of Public Health, Vietnam (IRB Nos. 498/2019/YTCC-HD3 and 312/2020/YTCC-HD3).

Informed Consent Statement: Written informed consent has been obtained from the stroke patients to publish this paper.

Data Availability Statement: The raw data supporting the findings of this article will be made available upon reasonable request to the corresponding authors.

Acknowledgments: We sincerely thank the support of experts and researchers, as well as the patients from six hospitals participating in this study.

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

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Mexican and Puerto Rican Men's Preferences Regarding a Healthy Eating, Physical Activity and Body Image Intervention

Lisa Sanchez-Johnsen [1,2,*], Amanda Dykema-Engblade [3], Carlos E. Rosas [1,2], Leonilda Calderon [4], Alfred Rademaker [5], Magdalena Nava [6] and Chandra Hassan [7]

[1] Department of Family and Preventive Medicine, Rush University Medical Center, 1645 West Jackson Blvd, Suite 302, Chicago, IL 60612, USA
[2] Department of Psychology, University of Illinois at Chicago, 1007 W Harrison St., Chicago, IL 60607, USA
[3] Department of Psychology, Northeastern Illinois University, 5500 North St. Louis Ave, Chicago, IL 60625, USA
[4] Puerto Rican Cultural Center, 2628 W. Division St., Chicago, IL 60612, USA
[5] Department of Preventive Medicine, Feinberg School of Medicine, Northwestern University, 680 N Lake Shore Drive, Suite #1400, Chicago, IL 60611, USA
[6] Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, 625 N. Michigan Ave Suite #1100, Chicago, IL 60601, USA
[7] Department of Surgery, University of Illinois at Chicago, 840 South Wood Street (MC 958), Chicago, IL 60612, USA
* Correspondence: lisa_sanchez-johnsen@rush.edu

Abstract: This study examined the logistical, practical, and cultural preferences of Latinos regarding the design of a healthy eating, physical activity, and body image intervention. Puerto Rican and Mexican men (n = 203) completed an interview as part of an NIH-funded study. Overall, 66.5% preferred the intervention to be in Spanish only or both Spanish and English; 88.67% said it was moderately, very or extremely important for the intervention leader to be bilingual; and 66.01% considered it moderately to extremely important for the leader to be Hispanic or Latino. Most participants (83.74%) reported they would be willing to attend an intervention that met twice per week and 74.38% said they would be willing to attend an intervention that met for 1.5 to 2 h, twice weekly. Overall, the majority said they would be moderately to extremely interested in attending an exercise program if it consisted of aerobics with Latin or salsa movements (74.88%) and if it consisted of aerobics with Latin or salsa music (70.44%). Some participants were moderately to extremely interested in attending an intervention if it included *dichos* (Latino sayings) (65.02%) and *cuentos* (folktales or stories) (69.46%). The findings have implications for lifestyle and body image interventions aimed at preventing cardiometabolic diseases.

Keywords: Latino men; obesity; intervention; diet; body image; physical activity

Citation: Sanchez-Johnsen, L.; Dykema-Engblade, A.; Rosas, C.E.; Calderon, L.; Rademaker, A.; Nava, M.; Hassan, C. Mexican and Puerto Rican Men's Preferences Regarding a Healthy Eating, Physical Activity and Body Image Intervention. *Nutrients* **2022**, *14*, 4634. https://doi.org/10.3390/nu14214634

Academic Editors: Silvia V. Conde and Alessandro Sartorio

Received: 30 August 2022
Accepted: 26 October 2022
Published: 3 November 2022

1. Introduction

Among men, overweight (BMI $\geq$ 25) and obesity (BMI $\geq$ 30) are serious health issues that are associated with numerous health problems, including cardiometabolic diseases [1–4]. Latino men, in particular, have concerningly high rates of overweight or obesity. In fact, Hispanic/Latino men have the highest prevalence (41.2%) of obesity or severe obesity compared to non-Hispanic Black (37.5%), non-Hispanic White (36.9%), and Asian (11.7%) men [5]. Moreover, there are marked differences in overweight and obesity rates between Latino men of different backgrounds. In the *Hispanic Community Health Study/Study of Latinos* (HCHS/SOL), a landmark epidemiologic study of Hispanics/Latinos, men of Puerto Rican (40.9%) and Mexican (36.8%) descent—the two largest Hispanic/Latino subgroups in the U.S. [6]—had the highest rates compared to men of other Hispanic/Latino backgrounds [7]. The differences in the prevalence of overweight and obesity between Hispanics/Latinos and other racial/ethnic groups and between different Hispanic/Latino ethnic groups are attributable to behavioral and environmental factors in addition to genetic

influences [8,9]. Given the influential role of obesity in development of cardiometabolic diseases [3], addressing these disparities is critical. Behavioral weight loss interventions are the preferred method for treating overweight and obesity; however, few weight loss interventions have been developed specifically for Latino men [10,11] and fewer, if any, have focused on specific subgroups of Latinxs.

The majority of diet and physical activity interventions conducted with Hispanic/Latinos have primarily targeted women, e.g., [12–16]; and only a few, to our knowledge, have focused exclusively on Hispanic/Latino men [17,18]. Indeed, the dearth of physical activity interventions for Hispanic/Latino men has been noted in two recent systematic reviews [10,11]. The low number of Hispanic/Latino men in weight-loss interventions is surprising given that studies indicate that, in general, men are interested in interventions that address overweight and obesity [19].

Despite this interest, men tend to enroll less frequently in behavioral weight loss interventions than women [20], and this disparity is exacerbated among Hispanic/Latino men [21,22]. Lack of enrollment of Latino men in weight loss interventions is two-sided. On one hand, Latino men face logistical (e.g., limited off-work time) and sociocultural (e.g., lack of control of diet due to gender roles) barriers that make it difficult to adopt healthy lifestyle behaviors [21,22]. For example, in a study among 14 Latino men with overweight, Garcia et al. [21] found that some of the barriers to healthy eating and physical activity included dietary norms and familial influence, long work hours, and fatigue. On the other hand, there is a lack of interventions that are designed to address the specific preferences and needs of Latino men [23]. While some reasons for the lack of enrollment—such as limited off-work time—among men are similar across racial/ethnic groups, others are specific to Hispanic/Latino men (e.g., spoken language, living environment, social, cultural, and familial influences) [21,23]. Factors that may be important to address these needs and overcome some barriers include the language of the intervention and program materials, the characteristics of the intervention leader, and the location and type of physical activity [21,24], and the integration of cultural elements into the intervention.

Moreover, interventions that focus on Latino men are important due to their patterns of diet and physical activity. For example, in the HCHS/SOL, individuals of Puerto Rican descent had the lowest estimated intake of fruits and vegetables compared to other Hispanic/Latino groups [25]. Further, a large proportion of Hispanic/Latino men (>73%) do not engage in regular physical activity [26]. Data from a recent study revealed that number of hours watching television per day was higher among Puerto Rican than Mexican men [27]. In another study, 47.5% of Mexican men and 56.9% of Puerto Rican men reported no leisure-time physical activity [28].

According to recent U.S. Census data, 67.6% of Hispanics/Latinos speak Spanish at home, and of those, 22.8% do not speak English well [29]. Language preference, however, varies by nativity and Hispanic/Latino background, with U.S.-born and Puerto Ricans more likely to speak English only than foreign-born and other Hispanics/Latinos [30]. Thus, an English- or Spanish-only intervention may not be appropriate for all Hispanic/Latino groups. Moreover, some Latinx groups (e.g., less acculturated groups) may prefer a Latinx intervention leader whereas others may not have a specific preference for the ethnicity of the leader. In a past qualitative study, a sample of 39 Mexican American women and men expressed that the ideal physical intervention would be led by a bilingual instructor [31]. However, no study, to our knowledge, has examined the preferences regarding the ethnicity of intervention leaders among Latinx men.

Another important consideration for interventions is the location of the intervention. Indeed, inadequate access to or lack of safe spaces for physical activity has been identified as a major barrier among Hispanic men [18,23,31]. Hispanic men report that their neighborhoods may be unsafe (e.g., gang activity) or inadequate (e.g., lack sidewalks and/or streetlights) for exercise, but, at the same time, transportation to and from facilities outside of their communities may be time-consuming [18,23,31]. While past studies have

assessed Latinx men's perceived barriers, few, if any, have inquired about their preferences. Relatedly, preferences for specific types of physical activity reported in previous studies are mixed, with some Latino men preferring team sports [18,32] and others walking [31]. Despite that dancing-based aerobic exercises are easier to implement (e.g., can be performed in- or out-doors, requires less space) than other forms of physical activity and are popular among Latinx men [33], little is known about Latinx men's preferences regarding aerobics-based interventions.

Culture-specific content are also important to include in interventions that address diet and physical activity among culturally and linguistically diverse populations [34]. One type of cultural-specific content is *dichos*, or Latino sayings or proverbs. Although *dichos* have not been used in a combined diet, physical activity and body image intervention for Latino men, they have been used in counseling [35,36], educational or health literacy programs [37,38], and in other interventions among Latinos [39–41]. For instance, *dichos* have previously been used in the development of an intervention focused on nutrition for Latinas and their families [42], and in another one focused on diet, physical activity, body image, and secondhand smoke intervention with Latina women [43]. Specifically, *dichos* can be validating to a person, can communicate openness to a person's Latino culture, and can be used to build rapport and trust [35]. Thus, *dichos* may be a culturally appropriate strategy to recruit, improve communication, and encourage healthy lifestyle behaviors among Latinos [38]. It is unknown, however, whether including *dichos* in an intervention would make it more appealing for Latino men. Similarly, the use of *cuentos*—folktales or stories/storytelling in Spanish—has yet to be examined in a health intervention with Latino men. Although not used extensively, *cuentos* have also been used in therapy and interventions to promote personal and social development and healthy behaviors [44–46]. If *dichos* and *cuentos* are important for Latino men, then such cultural components may be used to guide the development of a diet, physical activity and body image intervention for Latino men.

Latino men have been largely absent from interventions seeking to promote healthy lifestyles, and it is possible that this omission is due to the lack of tailoring interventions to their specific needs and preferences. Moreover, few studies, if any, have assessed differences in needs and preferences among different Latinx groups. To this end, the present study aimed to examine the preferences of Mexican and Puerto Rican men regarding the design and development of a healthy eating, physical activity and body image intervention. Specifically, the following were examined: Preferences regarding the language of the intervention and intervention materials, the importance of the intervention leader's language and Hispanic or Latino background; preferences regarding the frequency, duration, and location of the intervention; preferences regarding the type of exercise program; and questions regarding the use of various cultural elements such as *dichos* and *cuentos* during the intervention.

2. Methods

2.1. Sample

Data for the present study comes from the Latino Men's Health Initiative, which was an NIH-funded cross-sectional study (R21CA143636) designed to explore the role of cultural variables underlying race and ethnicity (acculturation, acculturative stress, ethnic identity, and cultural values) as they related to diet, physical activity, and body image among normal weight, overweight and obese Mexican and Puerto Rican men [47]. The Latino Men's Health Initiative relied on the heavy involvement of community partners who work with Latinos for a complete description, see [47]. Relying on community partners' experiences and expertise is consistent with the community-based participatory research (CBPR) model and allows for community partners to be involved in all aspects of the research process (e.g., recruitment, designing of interventions, dissemination of results) [48]. Data collection for the overall parent study (Latino Men's Health Initiative) was funded between 2010 and 2014. Overall, results from the Latino Men's Health Initiative will be used to

gain insight into the best ways to develop diet, physical activity and body image interventions for Mexican and Puerto Rican men using community-engaged and culturally targeted approaches.

Two hundred three Latino men (99 Mexicans and 104 Puerto Ricans) participated in the study. As noted elsewhere [47], the sample consisted of the following weight categories: Mexicans: 31.31% (n = 31) normal weight (BMI = 18.5–24.99), 36.36% (n = 36) overweight (BMI = 25–29.99), and 32.32% (n = 32), obese (BMI $\geq$ 30); Puerto Ricans: 35.58% (n = 37) normal weight, 32.69% (n = 34) overweight, and 31.73% (n = 33) obese.

Inclusion criteria were as follows: (a) Self-identified Mexican or Puerto Rican men, even if they identified as biracial. (b) Ages 18–65. (c) Those who agreed to provide informed consent. Exclusion criteria were as follows: (a) Men with a BMI lower than 18.5 kg/m^2 (no upper BMI limit). (b) Men who were unable to speak/read English or Spanish. (c) Those with an eating disorder (Bulimia Nervosa, Anorexia Nervosa, Binge Eating Disorder). (d) Men who had the intention to leave the Chicagoland area over the course of the study (i.e., 6 weeks).

2.2. Recruitment

The current study utilized both direct and indirect forms of recruitment. Direct recruitment involved distributing flyers at Hispanic/Latino community organizations, churches, festivals, and health fairs. The flyers promoted a free culture and health research study for Latino males. Additionally, flyers were provided to health care professionals, community members and posted in various locations throughout Chicago. Indirect recruitment consisted of newspaper advertisements, newsletters, letters to doctors and other organizations focused on Latino and/or health issues, postings to email listserves and websites, and at the University of Illinois at Chicago. For a thorough description of the recruitment strategies, see Sánchez-Johnsen et al. [47].

2.3. Study Procedures

Oral consent was obtained prior to determining an individual's initial eligibility. Once oral consent was obtained, research assistants proceeded to administer a 30 min eligibility interview via telephone or in-person. For those who met the initial eligibility criteria, a face-to-face visit was scheduled to complete the written consent. After written informed consent was obtained in-person, height and weight were measured using a stadiometer and Seca company digital scale, respectively. Body Mass Index [(BMI) weight (kg)/height (m)^2)] [49] was calculated to determine eligibility. As part of the larger NIH-funded study (R21CA143636), eligible participants completed a two and a half hour "Health and Culture" interview, which assessed their demographic and background characteristics, health and diet choices, weight, culture (e.g., cultural values and beliefs), exercise patterns, body image, and social support questions, and preferences about the development of a future intervention focused on healthy eating, physical activity, and body image See [47] for additional details. To assess participants' preferences regarding the development of a future intervention, they were given the following instructions: "At some point in the future, we will be developing a healthy eating, physical activity, and body image program that will focus on Latinos. In the next part of the interview, we will ask about your opinions and preferences concerning the development of a healthy eating, physical activity, and body image intervention in the future. Your responses will help us to identify certain things that may need to be considered when developing these programs in the future". Twenty percent of the overweight and obese sample were also asked to complete an in-depth qualitative interview adapted from [50]. Participants were also asked to complete a diet questionnaire and to use an accelerometer for seven days and record the results. The following additional objective measures were obtained: Bioelectrical Impedance Analysis, using a Tania BF 682 [51] to assess body fat, and waist and hip circumference, to assess fat distribution.

Participants were compensated for completing the health and culture interview ($50) and the other components of the study ($10 for completing the diet questionnaire, $15 for

using the accelerometer and recording the results, and $40 for completing the qualitative interview). The Institutional Review Board at the University of Illinois at Chicago (IRB 2011-0187) and the Research Review Board at Alivio Medical Center approved this study.

2.4. Community Partners

In the best effort to meet the needs of community members, the first author collaborated with community partners who served as members of the Hispanic/Latino Health Community Advisory Board (HLH-CAB). Prior to recruitment, HLH-CAB members assisted with the development of the recruitment plan and study methods, as well as provided critical insights and feedback about the content and design of the questionnaires, interview materials, informed consent document, incentives, and objective measures details can be found in [47]. Overall, along with the study team, the HLH-CAB sought to conduct the study at the highest level of cultural proficiency and to ensure that the study was relevant to the Mexican and Puerto Rican communities, while paying attention to differences within and among Latino communities.

2.5. Measures

2.5.1. Translation of Measures

All study materials were available in English and Spanish. Measures not available in Spanish were translated as recommended by numerous authors, e.g., [52,53]. Translated materials were then reviewed by members of the HLH-CAB and bilingual research assistants through a community engaged research process See [54] for additional information about the translation process.

2.5.2. Demographics

Demographic information, including information about their specific Latino background, race, age, marital status, education, occupation, and nativity, was self-reported. Descriptor variables included in the study were questions assessing demographics, literacy level, socio-economic status [55,56], and various additional questions assessing inclusion and exclusion criteria, e.g., [57]

2.5.3. Intervention Language and Ethnicity of Intervention Leader

Questions were posed about the design of a future intervention focused on healthy eating, physical activity and body image. Participants were asked about their language preferences regarding an intervention [language of the intervention and language of the intervention materials (e.g., handouts, instructions), respectively]. Response choices included: (1) Spanish only, (2) English only, (3) Both Spanish and English or (4) Other. Participants were also asked questions about the importance of the intervention leader to be Hispanic or Latino and the importance of the leader to be bilingual. Response choices were (1) not at all important, (2) not very important, (3) moderately important, (4) very important, or (5) extremely important.

2.5.4. Structure of Intervention

Questions were also asked about the frequency and duration of the intervention. Specifically, participants were asked, "Would you be willing to attend an intervention that met twice per week?" and "Would you be willing to attend an intervention that lasted for about one and a half to two hours on the first day and one and a half to two hours on the second day? Response choices for each question were "yes" or "no". Participants were also asked to rate their interest in attending an intervention if it was located in certain areas. Three of the four areas included in the questionnaire are located in Chicago neighborhoods that are traditionally considered as comprising large Mexican (Pilsen, Little Village) and/or Puerto Rican (Humboldt Park) communities. In addition, participants were asked how interested they would be to attend an intervention at University of Illinois at Chicago (the

university where the study PI was a faculty member at that time). Interest was rated on a 5-point Likert scale, which ranged from (1) Not at all interested to (5) Extremely interested.

2.5.5. Exercise Program

Preferences regarding the type of exercise program was also assessed. Participants were asked how interested they would be to attend an exercise program if the exercise consisted of aerobics with Latin or salsa music, or if the exercise consisted of aerobics conducted with Latin or salsa movements. Response choices ranged from (1) not at all interested to (5) extremely interested on a 5-point Likert scale.

2.5.6. Integration of Cultural Elements

Finally, questions were asked about the inclusion of cultural elements called *dichos* (Latino sayings or proverbs) and *cuentos* (stories or storytelling). Participants were first asked whether they have heard of *dichos*. If they responded yes, then they were asked if they have heard of *dichos* related to exercise, physical activity, or dancing; and whether they have heard of *dichos* related to their body or to health, respectively. They responded either "yes" or "no". Participants were then asked how interested they would be in participating in an intervention if it included *dichos*, in order to make it more culturally meaningful for Latinos. Response choices ranged from (1) Not at all interested to (5) Extremely interested on a 5-point Likert scale.

Similarly, participants were first asked whether they had heard any *cuentos* (stories or story-telling). If they responded affirmatively, they were then asked whether any of those cuentos related to exercise, physical activity, or dancing; and whether they have heard of *cuentos* related to their body or to health, respectively. After those questions, participants were asked how interested they would be in participating in an intervention that included *cuentos*, in order to make it more meaningful for Latinos. Participants were asked to respond to these questions using a 5-point Likert scale, which ranged from (1) not at all interested to (5) extremely interested.

2.6. Statistical Analyses

Frequencies and percentages were computed for sociodemographic factors. Responses were compared between the Mexican and Puerto Rican groups using the chi-square test of independence. Data were first analyzed using the overall sample of all weight groups. Given our interest in designing an intervention for individuals with overweight or obesity, a second analysis compared the groups restricting the analyses to those who were overweight and obese (BMI $\geq$ 25 kg/m^2). SPSS was used for all analyses and statistical significance was indicated if $p < 0.05$.

3. Results

3.1. Sample Characteristics

Demographic and background characteristics have been reported elsewhere [47] and summarized here. Four-hundred and thirty-five participants contacted members of the research team, 344 completed the oral script and initial eligibility interview, 211 men completed the written consent, 203 men completed the "Health and Culture" interview, and 193 completed all study components which included the "Health and Culture" interview, the diet questionnaire, and the use of an accelerometer. The data reported here are from the 203 Latino men (99 Mexican and 104 Puerto Ricans) who completed the "Health and Culture" interview. Participants' ages ranged from 18 to 65 years (M = 39.4, SD = 12.3). Most were single/never married (52.7%; n = 107) and had a high school diploma or equivalent (GED) or higher or some college (1–3 years) or graduated from a 4 year college (66.0%, n = 134). As intended, participants were roughly equally distributed between the three weight categories: 31.5% Normal, 34.5% Overweight, and 32.0% Obese.

There were significant differences in the following variables: age, with Puerto Rican being older ($p < 0.0001$), less likely to be single/never married ($p < 0.05$), more likely to have

been born in the U.S. mainland ($p < 0.05$), more likely to smoke ($p < 0.05$), and more likely to be unemployed ($p < 0.001$) than Mexicans. Other characteristics including the highest grade completed, the percent of men who had health insurance, religion and the language in which the interview was completed showed no significant differences between Puerto Ricans and Mexicans. Finally, there were no significant differences between Puerto Ricans and Mexicans in weight-related measures (i.e., BMI, body fat, hip and waist measurements).

3.2. Intervention Language and Ethnicity of Intervention Leader

As seen in Table 1, results revealed that 66.50% reported the language which they preferred the intervention to be conducted was Spanish or Spanish and English and 69.46% reported that the preferred language of the intervention materials was in both Spanish and English. Results revealed that 88.67% said it was "moderately", "very" or "extremely important" for the intervention leader to be bilingual and 66.01% said it was either "moderately", "very" or "extremely important" for the intervention leader to be Latino.

Table 1. Preferences for Language, Hispanic/Latino Background, Length, and Frequency of the Intervention among All Participants and among Participants with Overweight or Obesity.

Preferences: *n* (%)	Overall (*n* = 203)	All Puerto Ricans (*n* = 104)	All Mexicans (*n* = 99)	*p*	PR with Overweight/ Obesity (*n* = 67)	MX with Overweight/ Obesity (*n* = 68)	*p*
Language of the Intervention Spanish Only or Both Spanish and English	135 (66.50%)	70 (67.31%)	65 (65.66%)	0.80	44 (65.67%)	47 (69.12%)	0.67
Language of Intervention Materials Both Spanish and English	141 (69.46%)	71 (68.27%)	70 (70.71%)	0.71	43 (64.18%)	49 (72.06%)	0.33
Importance of Intervention Leader to be Bilingual [a]	180 (88.67%)	94 (90.38%)	86 (86.87%)	0.43	60 (89.55%)	59 (86.76%)	0.62
Importance of Intervention Leader to be Hispanic or Latino [a]	134 (66.01%)	74 (71.15%)	60 (60.61%)	0.11	47 (70.15%)	43 (63.24%)	0.39
Frequency of Meetings [b] Willing to attend an intervention that met twice per week	170 (83.74%)	87 (83.65%)	83 (83.84%)	0.97	54 (80.60%)	58 (85.29%)	0.47
Length of Intervention [b] Willing to attend an intervention that lasted for about one and a half to two hours on the first day and one and a half hours on the second day	151 (74.38%)	78 (75.00%)	73 (73.74%)	0.84	51 (76.12%)	51 (75.00%)	0.88

Note. [a] Moderately, Very, or Extremely Important. [b] % Yes. PR = Puerto Ricans; MX = Mexicans.

3.3. Structure of Intervention

In terms of the frequency and length of the intervention, results revealed that 83.74% said they would be willing to attend an intervention that met twice per week, and 74.38% said they would be willing to attend an intervention that met for one and a half to two hours on the first day and one and a half to two hours on the second day. There were no significant differences between Mexican and Puerto Rican men in terms of these responses. Results were similar in terms of level of responses and differences between ethnic groups when analyses were restricted to overweight and obese participants only (Table 1).

As seen in Table 2, participants reported they would be "moderately" "very" or "extremely interested" to attend an intervention if it were in the following Chicago neigh-

borhoods: Humboldt Park (75.37%), the University of Illinois at Chicago area (72.41%), Pilsen (48.28%), and Little Village (38.92%). More Puerto Ricans than Mexicans preferred the location to be in Humboldt Park ($p < 0.0001$); more Mexicans than Puerto Ricans preferred the University of Illinois at Chicago ($p = 0.009$), Pilsen ($p < 0.0001$) or Little Village ($p = 0.003$). These preferences were similar when the sample was restricted to overweight and obese participants only (Table 2).

Table 2. Preferences for the Location of the Intervention and Exercise Preferences among All Participants and among Participants with Overweight or Obesity.

Preferences: *n* (%)	Overall (*n* = 203)	All Puerto Ricans (*n* = 104)	All Mexicans (*n* = 99)	*p*	PR with Overweight/ Obesity (*n* = 67)	MX with Overweight/ Obesity (*n* = 68)	*p*
Location Preferences: *n* (%) [a]							
Humboldt Park	153 (75.37%)	94 (90.38%)	59 (59.60%)	<0.0001	61 (91.04%)	40 (58.82%)	<0.0001
University of Illinois at Chicago area	147 (72.41%)	67 (64.42%)	80 (80.81%)	0.009	39 (58.21%)	55 (80.88%)	0.004
Pilsen	98 (48.28%)	35 (33.65%)	63 (63.64%)	<0.0001	23 (34.33%)	46 (67.65%)	0.0001
Little Village	79 (38.92%)	29 (27.88%)	50 (50.51%)	0.003	17 (25.37%)	37 (54.41%)	0.0006
Exercise Preferences: *n* (%) [a]							
How Interested to attend an Exercise Program if the exercise consisted of aerobics with Latin or Salsa Movements	152 (74.88%)	84 (80.77%)	68 (68.69%)	0.047	57 (85.07%)	45 (66.18%)	0.011
How Interested to attend an Exercise Program if the exercise consisted of aerobics with Latin or Salsa Music	143 (70.44%)	78 (75.00%)	65 (65.66%)	0.14	53 (79.10%)	43 (63.24%)	0.042

Note. [a] Moderately, Very, or Extremely Interested. PR = Puerto Ricans; MX = Mexicans.

3.4. Exercise Program

As also seen in Table 2, 74.88% of men said they would be "moderately", "very" or "extremely interested" in attending an exercise program if it consisted of aerobics with Latin or salsa movements, with Puerto Ricans indicating a greater preference ($p = 0.047$). Overall, 70.44% of men said they would be "moderately" "very" or "extremely interested" in attending an exercise program if the program consisted of aerobics with Latin or salsa music with no difference between ethnic groups. Among the overweight and obese sample, Puerto Ricans indicated a greater preference for both Latin or salsa movements ($p = 0.011$) or music ($p = 0.042$) (Table 2).

3.5. Integration of Cultural Elements

As seen in Table 3, 80.30% reported that they heard of *dichos*. Moreover, 59.51% reported that they heard of *dichos* related to food or eating, 46.63% reported that they heard of *dichos* related to exercise, physical activity or dancing, 54.60% reported that they heard of *dichos* related to their body, and 56.44% reported that they heard of *dichos* related to health. Overall, 65.02% reported that they were moderately, very or extremely interested in attending an intervention if it included *dichos*. In the overall sample, 69.46% reported that they were moderately, very or extremely interested in attending an intervention if it included *cuentos*, with the Mexican sample indicating a greater preference ($p = 0.012$). Results were similar in terms of level of response and ethnic differences when the sample was restricted to overweight and obese participants only (Table 3). In addition, in the

overweight/obese sample, more Puerto Ricans than Mexicans reported that they heard of *dichos* related to exercise, physical activity or dancing ($p = 0.04$).

Table 3. Preferences for the Location of Intervention and Exercise among All Participants and Participants with Overweight/Obesity.

Cultural Elements: *n* (%)	Overall (*n* = 203)	All Puerto Ricans (*n* = 104)	All Mexicans (*n* = 99)	*p*	PR with Overweight/ Obesity (*n* = 67)	MX with Overweight/ Obesity (*n* = 68)	*p*
Heard of Dichos [a]	163 (80.30%)	78 (75.00%)	85 (85.86%)	0.052	52 (77.61%)	56 (82.35%)	0.49
Dichos Related to Food or Eating [a]	97 (59.51%)	44 (56.41%)	53 (62.35%)	0.44	31 (59.62%)	35 (62.50%)	0.76
Dichos Related to Exercise, Physical Activity, or Dancing [a]	76 (46.63%)	41 (52.56%)	35 (41.18%)	0.15	30 (57.69%)	21 (37.50%)	0.04
Dichos Related to your Body [a]	89 (54.60%)	43 (55.13%)	46 (54.12%)	0.90	29 (55.77%)	31 (55.36%)	0.97
Dichos Related to Health [a]	92 (56.44%)	45 (57.69%)	47 (55.29%)	0.76	32 (61.54%)	30 (53.57%)	0.40
How Interested in Participating in an Intervention if it included Dichos(sayings proverbs) in order to make it more culturally meaningful for Latinos [b]	132 (65.02%)	62 (59.62%)	70 (70.71%)	0.10	37 (55.22%)	46 (67.65%)	0.12
How Interested in Participating in an Intervention if it included Cuentos (stories or storytelling), in order to make it more culturally meaningful for Latinos [b]	141 (69.46%)	64 (61.54%)	77 (77.78%)	0.012	39 (58.21%)	53 (77.94%)	0.014

Note. [a] % Yes. [b] Moderately, Very, or Extremely Interested. PR = Puerto Ricans; MX = Mexicans.

4. Discussion

Results from this study offer important insights about how interventions that address healthy eating/diet, physical activity and body image may be tailored to Mexican and Puerto Rican men. Results revealed that over half of the participants (66.5%) preferred the intervention to be in Spanish or Spanish and English. In addition, 88.68% of participants indicated that it was "moderately", "very" or "extremely important" for the intervention leader to be bilingual and 66.01% said it was either "moderately" to "extremely important" for the intervention leader to be Latino. These findings highlight the importance of bilingualism among Mexican and Puerto Rican men. It is particularly important to note that although only two-thirds of participants preferred interventions to be in Spanish or Spanish and English and for the leader to be Latino, almost all participants indicated they would prefer a bilingual leader. Our findings replicate a previous study among Mexican Americans that identified having a bilingual intervention leader/instructor as a key component of the ideal physical activity intervention [31]. This suggests that, in interventions among Puerto Rican or Mexican men, having a bilingual leader is of paramount importance.

A high percentage of respondents indicated that 1.5–2 h meetings twice a week would be feasible. Previous qualitative research has found that some of the most significant barriers to physical activity among Latino men are busy work schedules and fatigue likely due to strenuous jobs [21,23,32]. Tailoring interventions to fit within Latino men's busy schedules can help with recruitment and retention. Our findings suggest that meeting for up to two hours twice a week to engage in physical activity may be an appropriate starting point for community interventions. Although we did not specifically assess preferences for a virtual or telehealth intervention, future interventions may wish to also consider this option, while meeting twice weekly.

In addition to time, location of intervention is critical. In this study, Puerto Rican and Mexican participants reported a preference for different Chicago neighborhoods. Understandably, the results indicated that intervention location preferences follow the general ethnic composition of the same neighborhoods. That is, Puerto Ricans preferred interventions to be held in Puerto Rican neighborhoods and Mexicans in Mexican neighborhoods. Participants may feel more comfortable participating in interventions held in their communities. Alternatively, it is possible that participants chose a location based on the distance between the location and where they live, work, or how close that site is located to other places they visit, regardless of the ethnic composition of the community. Distance travelled to an intervention would be crucial in maintaining participant intervention adherence to the intervention program. Moreover, intervention location may have implications for long-term sustainability as participants may continue engaging in physical activity at the location of the intervention. Indeed, lack of access to safe spaces for exercise has been identified as a barrier to physical activity among Latinos [21,31].

In terms of the type of exercise program, the Puerto Rican participants with overweight/obesity indicated a significantly greater interest in exercise programs with Latin or salsa movements and music. This finding underscores the often-overlooked diversity within the Latinx community. In this study, Puerto Ricans showed a greater preference towards exercise programs with salsa movements and music than Mexicans. Usually, salsa music is more associated with Puerto Ricans or Puerto Rican music [58]. However, musical preferences among Latinxs vary; thus, it may be important for aerobics-based physical activity interventions to include culturally appropriate music for the target Latinx subgroup [11]. It should be noted, however, that the present study focused on the preferences of Latino men regarding an exercise program which included aerobics. While interventions that have included aerobics or dancing have been successful [59,60], other types of activities should be considered. Walking, for example, seems to be the preferred type of physical activity among individuals of Mexican ancestry [31,61].

Slightly more than half of all participants reported that they would be interested in attending an intervention that included *dichos* or *cuentos*. These findings suggest that *dichos* and *cuentos* may still be an important tool to include in interventions, albeit not as significant as other aspects (e.g., language of leader). Alternatively, the lower-than-expected interest in *dichos* and *cuentos* may be due to the age distribution and/or generational status of the participants. We will explore these factors in future analyses. Despite the widespread use of *dichos* and *cuentos* among Latinxs, there is a dearth of studies examining their role on physical activity and diet interventions in this group. Miranda et al. [38] suggest that *dichos* may serve as mantras of motivation for adopting healthy behaviors; however, they cautioned that *dichos* may not result in pragmatic changes for all. *Cuentos* may serve a similar role. Additional research, however, is warranted to examine the role and effectiveness of both *dichos* and *cuentos* in lifestyle and body image interventions.

This study has a number of limitations. First, it must be kept in mind that the current study is exploratory, and, therefore, interpretation of the results should be made cautiously. Second, the participants of this study were recruited from neighborhoods in Chicago, IL, thus the representativeness of the sample is limited. Third, the acculturation level, ethnic identity, and other background characteristics, such as place of residence, nativity, years in the U.S., and marital status, were not examined in relation to these findings or controlled statistically. This is an important area of future investigation and future studies will seek to further explore these variables as it relates to intervention preferences of Latino men. Fourth, questions that were reported in this paper were posed in a closed-ended fashion. Our future work will explore the physical activity responses of Latino men using an open-ended format, which will add to a deeper understanding of the preferences of Latino men. Finally, although it is a strength that this study included data on Mexicans and Puerto Ricans—two of the largest Latinx groups in the U.S.—there is a tremendous amount of diversity within and between these and other Latinx groups (e.g., acculturation) that should also be kept in mind when developing interventions for Latino men.

These limitations notwithstanding, a number of strengths must be highlighted. First, this study focused exclusively on Mexican and Puerto Rican men, a greatly understudied group, with a high prevalence of overweight and obesity. Second, a rigorous community-based protocol for recruitment and data collection resulted in invaluable information on design and delivery strategies for gender- and culture-sensitive interventions. Finally, together with results from our prior studies, the current study adds important insights that may help with designing healthy eating/diet, physical activity, body image, and overall overweight and obesity interventions for Mexican and Puerto Rican men.

Despite the need to involve Latino men in diet and physical activity interventions, more is needed to increase recruitment, adherence, and engagement in this group. One size fits all interventions have not been successful, in part, because Latino men have their own gender- and ethnicity-related set of preferences, barriers, and motivators in regard to diet and physical activity. First, in terms of language preference, future interventions should consider including bilingual intervention leaders and conducting the intervention itself in both languages (Spanish and English). Second, in terms of the structure, the Mexican and Puerto Rican men in this study preferred the intervention to be held twice per week for one and a half to two hours. This may provide a starting point for developing healthy eating, physical activity, and body image interventions. This study also illustrated the importance of selecting intervention locations, exercise programs (aerobics with Latin or salsa movements and music), and cultural elements (use of *dichos* and *cuentos*) that matched the preferences of men in this study. Such considerations should be taken into account when designing and delivering a healthy eating, physical activity, and body image intervention among Mexican and Puerto Rican men. In conclusion, our study provides critical information about the intervention preferences of Mexican and Puerto Rican men that may be used to guide the design and development of Latinx-centered obesity interventions for men in an effort to reduce risk of cardiometabolic diseases.

Authors' Notes

Parts of this paper were presented as an oral presentation at the 8th Annual University of Illinois at Chicago Minority Health Conference, Chicago, Illinois, April 2017, and at the Fourth International Culturally Responsive Evaluation and Assessment Conference, Chicago, Illinois, September 2017. Parts of this paper were also presented as a poster at the Latino Health Symposium, Medical Organization for Latino Advancement (MOLA), Chicago, Illinois, November 2017, where it also received the Best Poster Award for a Research Project. This work was conducted when Magdalena Nava was at the Puerto Rican Cultural Center.

The words 'Hispanic', 'Hispanic/Latino', and 'Latinx' are used interchangeably, depending on the study being reviewed. 'Latinx' is used as a general inclusive term to refer to all persons of Hispanic/Latin American background. The term 'Latino' is used for participants in the current study, as that was the term used in recruitment and/or interviews in the parent study (the Latino Men's Health Initiative).

Author Contributions: Conceptualization, L.S.-J.; Data curation, L.S.-J.; Formal analysis, A.R.; Funding acquisition, L.S.-J., A.D.-E., M.N. and A.R.; Investigation, L.S.-J.; Methodology, L.S.-J.; Project administration, L.S.-J.; Resources, L.S.-J., L.C. and M.N.; Supervision, L.S.-J.; Writing—original draft, L.S.-J., A.D.-E., M.N., A.R. and C.H.; Writing—review and editing, L.S.-J., A.D.-E., C.E.R., L.C., A.R., M.N. and C.H. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the National Institutes of Health's National Cancer Institute, Grant numbers R21CA143636 and R1CA143636-S to Sanchez-Johnsen. Research reported in this publication was also supported, in part, by the National Institutes of Health's National Cancer Institute, Grant numbers U54CA202995, U54CA202997, and U54CA203000 to Sanchez-Johnsen, Amanda-Dykema-Engblade, Alfred Rademaker, Magdalena Nava, and Leonilda Calderon. Additional funding was provided by the Department of Psychiatry and the Office of the Vice Chancellor for Research at the University of Illinois at Chicago, the University of Illinois Cancer Center, the University of Illinois Hospital & Health Sciences System- Population Health Sciences Program to Sanchez-Johnsen. Additional services were provided by the UIC Center for Clinical and Translational Science (Funded by UL1TR000050). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Institutional Review Board Statement: The Institutional Review Board at the University of Illinois at Chicago (IRB 2011-0187) and the Research Review Board at Alivio Medical Center approved this study.

Informed Consent Statement: Written informed consent was obtained from all participants involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We extend our appreciation to all research participants as well as to our community partners at the Puerto Rican Cultural Center's Diabetes Empowerment Center, the Greater Humboldt Park Community of Wellness, Casa Central, Bickderdike Redevelopment Corporation, and Alivio Medical Center. The authors gratefully acknowledge the following community partners for their involvement in various phases of the study: Magdalena Nava, Leonilda Calderon, Jaime Delgado, Julia Escamilla, Susan Vega, Jose Luis Rodriguez, Illeana Gomez and Christy Prahl. The authors also extend their deep appreciation to all of the research staff, students and trainees who helped with this study, including the following individuals: Liliana Bolanos, Kelly Ortega, Eduardo Bastian, Regina Reina, Michelle Toledo, Angelica Alonso, Alejandra Onate, Erin Rodriguez, Christian Gomez, Francis Caparroso, John Capua, Andres Carrion, and Julius Lara. The authors extend their gratitude to Irma Rodas for her technical assistance in the preparation of this manuscript.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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Clustering of Health Risk Behaviors in Mexican and Puerto Rican Men: Results from the Latino Men's Health Initiative

Angelica Alonso [1], Carlos E. Rosas [2,3], Alfred Rademaker [4] and Lisa Sanchez-Johnsen [2,3,*]

[1] School of Public Health, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA
[2] Department of Family and Preventive Medicine, Rush University Medical Center, Chicago, IL 60612, USA
[3] Department of Psychology, University of Illinois at Chicago, Chicago, IL 60607, USA
[4] Department of Preventive Medicine (Biostatistics), Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
* Correspondence: lisa_sanchez-johnsen@rush.edu

Abstract: Engaging in multiple health risk behaviors simultaneously may increase the risk for cardiometabolic diseases. This study examined the prevalence and clustering of three health behaviors (physical activity, fruit and vegetable consumption, and smoking) among Latino men. The participants were 99 Mexican and 104 Puerto Rican men who participated in a study addressing culture- and obesity-related factors. The health behaviors were obtained from self-reported and anthropometric assessments through objective measurements. Among all participants, 5% had no health risk behaviors, 30% had one, 47% had two, and 18% had all three; their most common health risk behavior cluster was low physical activity and low fruit and vegetable consumption (28%). Among Puerto Rican men, 7% had no health risk behaviors, 24% had one, 51% had two, and 18% had all three; their most common health risk behavior cluster was current smoker and low fruit and vegetable consumption (28%). Among Mexican men, 3% had no health risk behaviors, 36% had one, 43% had two and 19% had all three; their most common health risk behavior cluster was low physical activity and low fruit and vegetable consumption (33%). The findings highlight the need for lifestyle interventions that target multiple health risk behaviors related to cardiometabolic diseases in Latinos.

Keywords: Hispanic; Latino; Latinx; health risk; diet; physical activity; smoking; men

Citation: Alonso, A.; Rosas, C.E.; Rademaker, A.; Sanchez-Johnsen, L. Clustering of Health Risk Behaviors in Mexican and Puerto Rican Men: Results from the Latino Men's Health Initiative. *Nutrients* **2022**, *14*, 4495. https://doi.org/10.3390/nu14214495

Academic Editors: Abeer M. Mahmoud and Shane Phillips

Received: 28 August 2022
Accepted: 17 October 2022
Published: 26 October 2022

1. Introduction

Latinxs experience a disproportionate burden of cardiometabolic diseases, including high rates of hypercholesterolemia, obesity, diabetes, and hypertension [1,2]. The risk for and development of cardiometabolic diseases such as cardiovascular disease and diabetes are strongly influenced by modifiable health behaviors, particularly smoking, unhealthy diet, and low physical activity [3,4]. Although more attention has been paid to single health behaviors and their association with health [5], individuals often engage in more than one unhealthy behavior simultaneously (e.g., smoking and physical inactivity) [5], which may further increase their risk for chronic diseases. Indeed, prior studies have shown that the risk of chronic diseases and mortality increases with a greater number of health risk behaviors (see Lacombe et al. [6] for a review).

Moreover, extant research suggests that engagement in different health behaviors varies by race or ethnicity and sex. Specifically, men from racial or ethnic minorities generally have less favorable health behavior profiles than women and non-Hispanic or non-Latino White men [1,7]. For example, in general, men tend to engage in risky health behaviors more frequently and have higher odds of engaging in multiple behaviors simultaneously than women [8–10]. However, despite being part of the largest ethnic minority group in the United States (U.S.) [11], Latino men have seldom been included in studies focusing on clusters of health behaviors. Examining how health behaviors cluster together among this group may be an important step in developing culturally tailored,

preventive interventions to improve cardiometabolic health, as changing simultaneously versus isolated occurring behaviors may require different strategies [12].

1.1. Health Behavior Clustering

Albeit not specifically among Latinxs, several studies have explored simultaneously occurring clusters of behaviors, primarily focused on smoking, drinking, poor diet, and low physical activity, which comprise the main behavioral determinants of chronic diseases [13]. Past studies have uncovered various clusters among these behaviors. For instance, using data from the U.S. National Health and Nutrition Examination Survey (NHANES) III, Berrigan et al. [7] identified 32 patterns of health behavior among a representative sample of non-Hispanic Whites, non-Hispanic Blacks, and Mexican Americans. The most common pattern was adhering to recommendations on alcohol and tobacco consumption while not adhering to recommendations for physical activity, dietary fat intake, or fruit and vegetable consumption [7]. While this was also the most common pattern among Mexican Americans, it was less prevalent (13.8%) than among non-Hispanic Whites (14.7%) and non- Hispanic Blacks (18.4%). A second pattern that was particularly more prevalent among Mexican Americans than other groups was non-adherence to physical activity and to fruit consumption recommendations and adherence to tobacco, alcohol, and vegetable recommendations. Berrigan et al. [7] also found that men, regardless of their race or ethnicity, were 2.6 times more likely to not adhere to all five recommendations (physical activity, fruit and vegetable consumption, alcohol, tobacco use, and dietary fat intake) than women.

Using data from the Aerobics Center Longitudinal Study (ACLS), Héroux et al. [14] identified two clusters of unhealthy behaviors among a primarily non-Hispanic White (95%) sample of 13,621 participants in the U.S. The first group was composed of individuals more likely to engage in smoking, alcohol use, unhealthy diet, and low physical activity, while the second one was more likely not to engage in any of the four unhealthy behaviors [14]. They also found that all behaviors were significantly associated with each other, such that engaging in one behavior was related to increased odds of engaging in another one. For instance, individuals with an unhealthy diet (relative to those with a healthy diet) were 2.45 times more likely to engage in low physical activity, 2.02 times more likely to smoke, and 1.61 times more likely to drink heavily [14].

Research studies from other countries further support for the clustering of health behaviors and the importance of considering sociodemographic differences. In a population-based study among Irish adults, for example, Conry et al. [15] found six different clusters of behaviors. Individuals in the healthy lifestyle cluster, characterized by non-smokers, high physical activity, healthy eating, and moderate alcohol use, tended to be women, older (65+ years), and of higher socioeconomic status (SES), while those in the mixed lifestyle cluster (non-smokers, moderate physical activity, and variable alcohol consumption) were more likely to be men, younger, and of low SES [15]. Similarly, using population-based data from 4238 German participants, Rabel et al. [16] identified three clusters of behaviors which varied by sex. The healthiest cluster (low to moderate drinking, favorable diet, moderate physical activity, and no smoking) was endorsed by women only, while the other two more heterogenous clusters were endorsed primarily by men ($\geq$71%). Collectively, these studies suggest the co-occurrence of health behaviors is common, but patterns vary by sociodemographic factors. Men, in particular, seem to be more likely to engage in multiple risky behaviors than women; however, few studies have focused exclusively on men, and fewer on Latino men [17,18].

1.2. Health Behaviors in Latinxs

Past research on health behaviors among Latinxs has yielded mixed results. For instance, while some studies using self-reported data have found that fewer Hispanics/Latinos engage in less physical activity than non-Hispanic Whites [19,20], studies using objectively measured data indicate that Hispanics/Latinos engage in higher physical activ-

ity levels than non-Hispanic Blacks and Whites [21,22]. Similarly, some studies indicate that Hispanics have better-quality diets than non-Hispanic Blacks and Whites [23,24], while others find the opposite [19,25]. Moreover, although previous studies have found that smoking is not as prevalent among Hispanics/Latinos as it is among other racial/ethnic groups [7,26,27], these studies have focused primarily on Hispanics/Latinos of Mexican descent e.g., [7]. These differences may be explained by variations in diet, physical activity levels, and smoking rates between Latinx subgroups. Indeed, evidence from the Hispanic Community Health Study/Study of Latinos (HCHS/SOL) has revealed differences in diet quality [28], physical activity [29], and smoking [18] across Hispanic/Latino subgroups; thus, research on specific Latinx subgroups is warranted. In addition, it is imperative to examine the specific clustering of health behaviors, as certain combinations appear to be related to different health conditions. One study among ethnically diverse adults, for example, reported that not eating a healthy diet along with not doing vigorous physical activity was associated with an increased risk for hypertension, whereas not eating a healthy diet alone was a stronger predictor of diabetes [30].

Despite the high prevalence of health risk behaviors in Latinxs, especially men, very few studies have examined risk behavior clustering in this population. Moreover, clustering patterns may differ across ethnic and sex groups, and this may provide insight into the development of more effective health behavior interventions. To this end, the overall objective of this study was to examine the prevalence and clustering of three important health behaviors (smoking, fruit and vegetable intake, and physical activity) among Mexican and Puerto Rican men. We also examined whether these associations varied by Latino background.

2. Materials and Methods

2.1. Sample

This is a secondary analysis of data obtained from Mexican and Puerto Rican participants who enrolled in a cross-sectional study (R21CA143636) called the Latino Men's Health Initiative (Iniciativa de Salud para Hombres Latinos). The Latino Men's Health Initiative was an NIH-funded, community-based participatory research study [17]. The purpose of that cross-sectional parent study was to explore the role of four cultural variables (acculturation, acculturative stress, ethnic identity, and cultural values) that may help to explain the ethnic disparities in correlates of overweight or obesity (diet, physical activity, and body image) among Mexican and Puerto Rican men [17]. Eligibility criteria were as follows: Inclusion: (a) Mexican and Puerto Rican men. They could be biracial but had to identify as Mexican or Puerto Rican. (b) Between the ages of 18 and 65. (c) Those who agreed to provide informed consent. Exclusion: (a) Those with a lower body mass index (BMI) limit: <18.5 kg/m^2. No upper BMI limit (as the target population of the larger study were men with normal weight, overweight, or obesity). (b) Those who were not able to comprehend English or Spanish. (c) Those who had an eating disorder, including bulimia nervosa, anorexia nervosa, and binge eating disorder. (d) Those who had plans to move from the Illinois area during the course of the study (i.e., 6 weeks).

The recruitment strategies, which are described in detail elsewhere [17], consisted of direct and indirect methods. The direct methods included in-person recruitment at Latinx organizations, churches, and community events. The indirect methods included newspaper and newsletter advertisements, website and listserv announcements, and letters to organizations with a Latino or health focus. All participants provided written informed consent. The data collected as part of the overall parent study and the analyses reported in the current study was approved by the Institutional Review Board (IRB) at the University of Illinois at Chicago (IRB-2011-0187). The parent study also received IRB approval from the research review board at Alivio Medical Center. In addition, the University of Illinois at Chicago served as the IRB of record for Northwestern University (STU00204427).

The participants completed anthropometric measures (height, weight, body fat, hip, waist), a health and culture interview, and a diet questionnaire and used an accelerometer

for seven consecutive days. As noted elsewhere [17], 203 participants completed the measures and the health and culture interview, and 193 completed all study components.

2.2. Measures

2.2.1. Demographics and Health-Related Variables

Sociodemographic, clinical, and health-related information was obtained from participants during a two and a half hour health and culture interview (see Sanchez-Johnsen et al. [17]). During the eligibility interview, the participants were asked, 'What is your ethnic background?' The response options included 'Hispanic or Latino (can you specify which Hispanic or Latino ethnic group)', along with 'African American or Black (Not Hispanic)'; 'White, Caucasian, or European (Not Hispanic)'; and five other categories. The participants were allowed to choose all that applied but had to specify that they were Mexican or Puerto Rican to continue participating in the study. In addition, the participants were asked to self-report their age, nativity, length of time lived in the U.S., highest grade completed, income, smoking status, and other socio-demographic background characteristics. For the purpose of this study, the length of time lived in the U.S. ($\leq$20 years, 21–40 years, and 41–65 years), education (less than high school graduate, some high school education, high school graduate or equivalent/GED, some college education, and college graduate of a four-year university or graduate studies), and income (<$10,000, $10,000–$24,999, $25,000–$49,999 and $\geq$$50,000) were treated as ordinal variables. BMI was included as a categorical variable (categories defined below), and age was treated as a dichotomous variable with two categories (18–40 and 41–65 years).

2.2.2. Body Mass Index

Weight status was assessed via BMI (weight (kg)/height $(m)^2$) [31]. After removing their shoes, the participants' height and weight were assessed using a SECA stadiometer (SECA, Chino, CA, USA) and SECA digital scale (SECA, Chino, CA, USA), which had been used in previous studies [32]. Weight status was defined as: normal weight: BMI = 18.5–24.99; overweight: BMI = 25–29.99; obese: BMI $\geq$ 30 [31].

2.2.3. Smoking Status

Smoking status was assessed using a single item from the Adult Stages of Change for Smoking questionnaire [33,34] which measured participants' stage of change for quitting smoking. Participants were asked to respond to the question, 'Do you currently smoke cigarettes?' The answer was treated as a dichotomous variable to maintain uniformity within our analysis. The two categories were recoded and defined by 'Yes' (currently smoke) and 'No' (never smoked or quit smoking).

2.2.4. Self-Reported Physical Activity

Physical activity was assessed via a single item from the Stages of Change for Exercise questionnaire [35,36], which measured participants' stage of change for increasing exercise. Participants were asked to respond to the question, 'Do you currently engage in regular physical activity?' The following instructions were included: 'For physical activity to be considered "regular" it must be done for 30 min at a time (or more) per day, and be done at least four days per week.' Additional information about how physical activity was defined can be found in the studies by Hellsten et al. [35] and Nigg [36]. The response options were 'Yes' and 'No'.

2.2.5. Fruit and Vegetable Intake

Fruit and vegetable consumption was assessed using a single item from the Stages of Change for Fruit and Vegetable Intake survey, which measured the participants' stage of change for increasing fruit and vegetable consumption [37–39]. The participants were asked to respond to the question, 'How many servings of fruits and vegetables do you usually eat each day?' The responses ranged from zero to six or more. In line with the

established guidelines [40,41], the fruit and vegetable intake variable was dichotomized into '<5 servings/day' or '≥5 servings/day' for the purpose of this study.

2.3. Statistical Analyses

The descriptive statistics provided frequencies and percentages of participant characteristics and health risk behaviors, which were compared between Latino subgroups using the chi-square test. A sample size calculation was performed to achieve 80% power for overall correlation analyses and for comparison analyses between Latino descent groups.

Statistical analysis models were derived from the study by Baruth et al. [12]. The prevalence rates of (1) current smoking status, (2) physical activity, and (3) fruit and vegetable consumption were computed in the overall sample and in each Latino subgroup. Next, the prevalence of multiple health risk behaviors was calculated by assigning a score of 1 (versus 0) to each for being a smoker, engaging in less than 30 min of physical activity per day, and consuming <5 servings/day of fruits and vegetables. The number of risk factors for each person was the sum of these individual scores (score range 0–3).

A series of hierarchical log-linear models using SAS/STAT CATMOD 15.2 (SAS Institute, Cary, NC, USA) were fit to the observed counts using maximum likelihood estimation. First, the main effects model was used with the three risk behaviors. The model assumed mutual independence among the three risk behaviors. Then, one or more two-factor interactions were added to the model to obtain the model with the least significant likelihood ratio *p*-value (best fit as indicated by the least difference between the observed and expected frequencies). Within this best fit model, the most significant interaction term (i.e., the interaction term with the lowest *p*-value) identified the most important risk factor cluster. Any interaction included in the final model was interpreted as reflecting dependence among the risk behaviors. The models were calculated for all Latinos and for each Latino subgroup. For Puerto Rican models, 0.5 was used in place of the zero frequency where no Puerto Rican participants smoked and consumed at least 5 servings of fruits and vegetables.

3. Results

3.1. Demographic Characteristics

The demographic characteristics are described in detail elsewhere [17] and the key variables are summarized here. The sample included 203 Latino men (99 Mexicans and 104 Puerto Ricans) aged 18–65 years (M = 39.4; SD = 12.3). Most participants had lived in the U.S. more than 20 years (74%; n = 149), had acquired some college education (50%; n = 101), had income levels either less than $10,000 (29%; n = 59) or between $25,000–$49,000 (30%; n = 61), and had overweight or obesity as indicated by their BMI (66%; n = 135). The distribution of covariates remained relatively uniform across the Latino subgroups, except for age. Specifically, there were more Puerto Rican men (62%) aged 41–65 years than Mexican men (30%) of the same age group. The demographic information is reported in Table 1.

Table 1. Characteristics of Latino men.

	Overall	**Puerto Rican**	**Mexican**	
Variable	***n* (%)**	***n* (%)**	***n* (%)**	***p***
Country of Birth	203	104 (51)	99 (49)	
U.S., except Puerto Rico	114 (56)	62 (60)	52 (53)	
Puerto Rico	42 (21)	42 (40)	0	
Mexico	47 (23)	0	47 (47)	
Age				<0.0001
18–40	108 (53)	39 (38)	69 (70)	
41–65	95 (47)	65 (62)	30 (30)	

Table 1. *Cont.*

	Overall	Puerto Rican	Mexican	
Variable	***n* (%)**	***n* (%)**	***n* (%)**	***p***
Length of U.S. Residence [a]				<0.0001
0–20 y	52 (26)	14 (14)	38 (39)	
21–40 y	96 (48)	44 (43)	52 (53)	
41–65 y	53 (26)	45 (44)	8 (8)	
Education [b]				0.09
Less than HS grad	18 (9)	6 (6)	12 (12)	
Some HS	29 (14)	21 (21)	8 (8)	
HS/HS Equivalent/GED	53 (26)	26 (25)	27 (27)	
Some College	59 (29)	29 (28)	30 (30)	
College Grad 4+	42 (21)	20 (20)	22 (22)	
Income [c]				0.66
<$10,000	59 (29)	32 (31)	27 (27)	
$10,000–$24,999	48 (24)	21 (21)	27 (27)	
$25,000–$49,999	61 (30)	31 (30)	30 (30)	
≥$50,000	34 (17)	19 (18)	15 (15)	
BMI Status [d]				0.79
Normal weight [e]	68 (34)	37 (36)	31 (31)	
Overweight [f]	70 (34)	34 (33)	36 (36)	
Obese [g]	65 (32)	33 (32)	32 (32)	

Note: [a] Length of U.S. residence: Two people did not respond and were not categorized. [b] Education: HS = high school; GED = general educational development/diploma. Education: Two participants chose the option 'Other' and were not categorized. [c] Income: One participant did not respond and was not categorized. [d] BMI = body mass index. [e] BMI: 18.5–24.99; [f] BMI: 25–29.99; [g] BMI ≥ 30.

3.2. Prevalence of Health Risk Behaviors

The prevalence rates of each individual risk behavior, as well as the prevalence of the total number of risk behaviors, are shown in Table 2. Of the total sample of 203 Latino male participants, 38% smoked, 47% engaged in no physical activity, and 93% consumed <5 servings/day of fruits and vegetables. When examining the prevalence of multiple risk behaviors, 5% of the sample had no health risk behaviors, 30% had one, 47% had two, and 18% had all three health risk behaviors.

Table 2. Prevalence of health risk behaviors among Latino men (*n* = 203).

	Overall	Puerto Rican	Mexican	
Health Risk Behavior	***n* (%)**	***n* (%)**	***n* (%)**	***p***
Smoking				0.01
Yes	77 (38)	48 (46)	29 (29)	
No	126 (62)	56 (54)	70 (71)	
Low Physical Activity [a]				0.24
Yes	96 (47)	45 (43)	51 (52)	
No	107 (53)	59 (57)	48 (48)	
Low Fruits and Vegetables Intake				0.48
Yes (<5 servings/day)	188 (93)	95 (91)	93 (94)	
No (≥5 servings/day)	15 (7)	9 (9)	6 (6)	

Table 2. *Cont.*

	Overall	Puerto Rican	Mexican	
Health Risk Behavior	***n* (%)**	***n* (%)**	***n* (%)**	***p***
# of Risk Behaviors				0.20
0	10 (5)	7 (7)	3 (3)	
1	61 (30)	25 (24)	36 (36)	
2	96 (47)	53 (51)	43 (43)	
3	36 (18)	19 (18)	19 (19)	

Note: [a] Currently do not engage in regular physical activity. # = Number.

As previously reported by Sanchez et al. [17], 46% of the 104 Puerto Rican men smoked. In addition, the results from this study showed that among Puerto Rican men, 43% engaged in no physical activity, and 91% consumed <5 servings/day of fruit and vegetables. When examining the prevalence of multiple risk behaviors, 7% of the Puerto Rican sample had no health risk behaviors, 24% had one, 51% had two, and 18% had all three health risk behaviors.

As noted in Sanchez et al. [17], 29% of the 99 Mexican men smoked. In addition, the results from this study showed that among Mexican men, 52% engaged in no physical activity and 94% consumed <5 servings/day of fruit and vegetables. When examining the prevalence of multiple risk behaviors, 3% of the Mexican sample had no health risk behaviors, 36% had one, 43% had two, and 19% had all three health risk behaviors.

3.3. Clustering of Health Risk Behaviors

The prevalence rates of the eight possible health risk behavior clusters are shown in Table 3. The most common health risk behavior cluster was engaging in low physical activity and consuming <5 servings/day of fruits and vegetables (28%), followed by smoking and consuming <5 servings/day of fruits and vegetables (19%) and engaging in all three health risk behaviors (18%). Latino men did not have any frequencies for the categories of smoker with low physical activity and greater than or equal to 5 servings of fruits and vegetables.

Table 3. Clustering of health risk behaviors among Latino men (n = 203).

# of Risk Behaviors	Smoking (Y/N)	Low Physical Activity (Y/N)	Low Fruits and Vegetables Intake (Y/N)	Observed Frequency *n* (%)	Expected Frequency under Mutual Independence	Expected Frequency under Final Best Fit Model
3	Y	Y	Y	36 (18)	35.3	37.1
2	N	Y	Y	57 (28)	55.3	55.9
2	Y	N	Y	39 (19)	38.0	37.9
1	N	N	Y	56 (28)	59.5	57.1
1	N	Y	N	3 (2)	5.4	3.0
1	Y	N	N	2 (1)	3.7	2.0
0	N	N	N	10 (5)	5.8	10.0
2	Y	Y	N	0 (0)	0	0

Note: Latino men did not have any frequencies for the categories of smoker with low physical activity and greater than or equal to 5 servings of fruits and vegetables. Among all Latino men, the majority had 2 health risk behaviors consisting of low physical activity and low intake of fruits and vegetables per day. The final model indicates that there is a dependence of fruit and vegetable consumption on physical activity (p = 0.08) and smoking (p = 0.13). # = Number. Y = Yes. N = No.

The prevalence rates of six possible health risk behavior clusters for Puerto Rican men are shown in Table 4. The most common health risk behavior cluster was engaging in smoking cigarettes and consuming <5 servings/day of fruits and vegetables (28%), followed by low physical activity and consuming <5 servings/day of fruits and vegetables (23%), and lastly engaging in all three health risk behaviors (18%).

Table 4. Clustering of health behaviors among Puerto Rican men (n = 104).

# of Risk Behaviors	Smoking (Y/N)	Low Physical Activity (Y/N)	Low Fruits and Vegetables Intake (Y/N)	Observed Frequency n (%)	Expected Frequency under Mutual Independence	Expected Frequency under Final Best Fit Model
3	Y	Y	Y	19 (18)	19.2	21.7
2	N	Y	Y	24 (23)	22.0	21.3
2	Y	N	Y	29 (28)	25.1	26.3
1	N	N	Y	23 (22)	28.7	25.7
1	N	Y	N	2 (1.9)	2.3	2.2
0	N	N	N	7 (6.7)	3.0	6.8

Note: Among Puerto Rican men, the majority had 2 health risk behaviors consisting of smoking and low fruit and vegetable servings per day. The final model indicates that there is a dependence of fruit and vegetable consumption on both smoking (p = 0.04) and physical activity (p = 0.04). # = Number. Y = Yes. N = No.

The prevalence rates of seven possible health risk behavior clusters for Mexican men are shown in Table 5. The most common health risk behavior cluster was engaging in low physical activity and consuming <5 servings/day of fruits and vegetables (33%), followed by engaging in all three health risk behaviors (17%) and lastly engaging in smoking and consuming <5 servings/day of fruits and vegetables (10%).

Table 5. Clustering of health risk behaviors among Mexican men (n = 99).

# of Risk Behaviors	Smoking (Y/N)	Low Physical Activity (Y/N)	Low Fruits and Vegetables Intake (Y/N)	Observed Frequency n (%)	Expected Frequency under Mutual Independence	Expected Frequency under Final Best Fit Model
3	Y	Y	Y	17 (17)	17.81	14.56
2	N	Y	Y	33 (33)	33.00	33.85
2	Y	N	Y	10 (10)	10.18	13.41
1	N	N	Y	33 (33)	32.01	31.18
1	N	Y	N	1 (1)	1.81	2.60
1	Y	N	N	2 (2)	1.01	1.03
0	N	N	N	3 (3)	3.18	2.38

Note: Among Mexican men, the majority had two health risk behaviors consisting of low physical activity and low fruit and vegetable servings per day. The final model indicates that there is a dependence of fruit and vegetable consumption on physical activity (p = 0.21). # = Number. Y = Yes. N = No.

3.4. Modeling Results

The first log-linear model for Latino men assumed that the three risk behaviors were mutually independent of one another with a goodness of fit of χ^2 (3) = 5.0, p = 0.17. The final (best fit) log-linear model containing smoking, fruit and vegetable intake, physical activity, the fruit and vegetable intake by physical activity interaction and the fruit and vegetable intake by smoking interaction had an improved goodness of fit of χ^2 (1) = 0.11, p = 0.74. The most significant interaction term in the final model was the low physical activity by consuming <5 servings/day of fruits and vegetables interaction (p = 0.08), which corresponded to the most frequent cluster in the overall sample (28%). For Latino men in general, of the individuals who had less than 5 servings of fruit and vegetables per day (n = 188), 40% smoked and 49% did not engage in physical activity, while of the individuals who had 5 or more servings of fruit and vegetables per day (n = 15), 13% smoked and 20% did not engage in physical activity. Table 3 presents the observed frequencies and the expected frequencies under the independence and final models.

For Puerto Rican men, the mutually independent model had a goodness of fit of χ^2 (4) = 10.1, p = 0.039. For the final (best fit) log-linear model containing smoking, fruit and vegetable intake, and physical activity, the fruit and vegetable intake and physical activity interaction and the fruit and vegetable intake and smoking interaction had an

improved goodness of fit of χ^2 (2) = 1.6, p = 0.45. The most significant interaction term in the final model was the smoking and consuming <5 servings/day of fruits and vegetables interaction (p = 0.04), which corresponded to the most frequent cluster in the Puerto Rican sample (28%). Of the individuals who had less than 5 servings of fruit and vegetables per day (n = 95), 45% did not engage in physical activity and 51% smoked, while of the individuals who had 5 or more servings of fruit and vegetables per day (n = 9), 22% did not engage in physical activity and 0% smoked. Table 4 presents the observed frequencies and the expected frequencies under the independence and final models.

For Mexican men, the mutually independent model had a goodness of fit of χ^2 (3) = 3.6, p = 0.21. For the final (best fit) log-linear model containing smoking, fruit and vegetable intake, and physical activity, the fruit and vegetable intake and physical activity interaction had an improved goodness of fit of χ^2 (2) = 1.6, p = 0.46. The most significant term in the final model was the low physical activity and consuming <5 servings/day of fruits and vegetables interaction (p = 0.21), which corresponded to the most frequent cluster in the Mexican sample (33%). For Mexican men, of the individuals who had less than 5 servings of fruit and vegetables per day (n = 93), 54% did not engage in physical activity, while of the individuals who had 5 or more servings of fruit and vegetables per day (n = 6), 17% did not engage in physical activity. Table 5 presents the observed frequencies and the expected frequencies under the independence and final models.

4. Discussion

We examined the prevalence and clustering of three common health behaviors among Mexican and Puerto Rican men. Our results revealed a high prevalence of smoking (38%), engaging in low levels of physical activity (47%), and particularly low consumption of fruits and vegetables (93%) among all participants. Among all participants, almost half (47%) engaged in two health risk behaviors while 30% engaged in one health risk behavior and 18% in all three health risk behaviors; only 5% did not engage in any health risk behavior. We also found differences based on Latino background. Specifically, fewer Puerto Rican (43%) relative to Mexican (52%) men engaged in low physical activity, although this difference was not significant. Lastly, more Puerto Ricans (51%) reported engaging in two health risk behaviors than Mexicans (43%); this difference, however, was not statistically significant.

These results align with the findings of previous studies. For example, the results of the HCHS/SOL revealed that the smoking rates were the highest (35.0%) among men of Puerto Rican descent compared to men of other Hispanic/Latino backgrounds [18]. Similarly, data from the Centers for Disease Control and Prevention demonstrated that between the periods of 2002–2005 and 2010–2013, the prevalence rates of smoking were the highest among Puerto Rican men (33.9%) compared to men of other Hispanic/Latino subgroups (22.5–27.6%), non-Hispanic White men (28.1%), and non-Hispanic Black men (31.7%) [42]. In addition, data from the HCHS/SOL showed that almost 50% of the men in the study engaged in less than 150 min per week of moderate to vigorous physical activity, with men of Puerto Rican descent having more minutes per day of moderate to vigorous physical activity, as assessed by an accelerometer, than men of other Hispanic/Latino backgrounds [29]. The previous studies have shown that Hispanics/Latinos do not meet the national recommendation for the consumption of fruits and vegetables [40,43,44], and the results of the HCHS/SOL revealed that individuals of Puerto Rican descent tend to eat less fruit and vegetables than other Hispanics/Latinos [45]. Overall, our findings provide further evidence of the high prevalence of smoking, physical inactivity, and low fruit and vegetable consumption among Mexican and Puerto Rican men.

Few studies have examined the prevalence of multiple risk behaviors among Latinxs; however, the few that have done so indicate that Hispanics/Latinos tend to engage in at least two risk health behaviors simultaneously [1,7]. In our study, almost half of all participants—and half of all Puerto Ricans—engaged in two health risk behaviors. These findings are somewhat similar to those of Daviglus et al. [1], which found that about

half of all Hispanic/Latino men had at least two cardiovascular disease risk factors. Although Berrigan et al. [7] assessed adherence to recommendations rather than health risk factors, they found that the most common pattern among all participants, including non-Hispanic Blacks, non-Hispanic Whites, and Mexican Americans, involved non-adherence to health behavior recommendations regarding physical activity, fat intake, or fruit and vegetable consumption.

We also identified specific clusters of health risk behaviors. For instance, Latino men, in general, who did not engage in "regular" physical activity (i.e., 30 min or more of physical activity per day for at least four days per week) were more likely to consume <5 fruits and vegetables per day. The most common health risk behavior cluster in Puerto Rican men was engaging in smoking cigarettes and consuming <5 servings/day of fruits and vegetables (28%), whereas the most common health risk behavior cluster among Mexican men was engaging in low physical activity and consuming <5 servings/day of fruits and vegetables (33%). Furthermore, in Puerto Rican men, smoking was associated with less fruit and vegetables intake per day. These risk clusters were also verified through multivariate modelling.

To our knowledge, no study to date has examined health behavior clustering exclusively among Latinxs. The only study that included Latinxs in their sample—albeit only Mexican Americans—produced similar findings to ours, with clusters of adherence to recommendations for alcohol and tobacco but non-adherence to recommendations for physical activity, dietary fat intake, and fruit and vegetable consumption [7]. In the HCHS/SOL, the most common patterns of any two cardiovascular disease risk factors were hypercholesterolemia and obesity followed by hypercholesterolemia and smoking among Hispanics/Latinos of a Puerto Rican background; and hypercholesterolemia and obesity followed by hypercholesterolemia and hypertension among Hispanics/Latinos of Mexican descent [1]. Those patterns are roughly consistent with the patterns found in the current study, wherein smoking was more prevalent among Puerto Ricans and physical inactivity among Mexicans. Daviglus et al. [1], however, did not examine the clustering of health behaviors.

The awareness of health risk behaviors among Latino men has implications for health and health promotion. The low levels of physical activity and high prevalence of smoking coupled with low intake of fruits and vegetables are major risk factors for morbidity and mortality [3,46]. Conversely, higher consumption of fruits and vegetables along with nuts and whole grains has been associated with lower cardiovascular disease-related morbidity and mortality in men [47]. Hence, our findings underscore the need for interventions that target multiple health risk behaviors simultaneously. Although interventions that target multiple co-existing health risk behaviors are needed and are believed to have the potential for a greater public health impact [48], they are scant among Latinxs. Their scarcity may be due to lack of research on behavior clusters in this population. Our findings may provide a starting point for such interventions. The differences in behavior clusters between Mexican and Puerto Rican men identified in this study, for instance, may aid in tailoring interventions to the specific needs of these groups, thereby increasing their effectiveness.

A few limitations must be borne in mind. First, in this secondary data analyses, measures for physical activity, smoking, and fruit and vegetable intake were cross-sectional and self-reported. Indeed, past studies on physical activity have yielded conflicting results when using self-reported versus accelerometer data (e.g., [20,22]). In addition, twenty four hour food recalls and food diaries are more effective methods to obtain accurate measurements of fruit and vegetable intake [49]. Second, our study was limited to Mexican and Puerto Rican men from the Chicagoland area, which limits the generalizability to other Latinx subgroups and Mexicans and Puerto Ricans in other cities. At least one study has found differences in health risk behaviors between Mexicans and Puerto Ricans and their counterparts in different cities across the U.S. [18]. Future studies should address these limitations by using objective methods across multiple time points.

These limitations notwithstanding, our study had a number of strengths. The most important strength of this study is its focus on a greatly underserved and understudied group, namely Latino men, and particularly Mexican and Puerto Rican men—the two largest Hispanic/Latino ethnic groups in the U.S. [11]. This is important not only because of the scant research on this group and its large population size, but also because of the high rates of engagement in health risk behaviors and high prevalence of overweight and obesity. A second strength is the focus of the current study on the differences between Latino subgroups. Few studies outside of the HCHS/SOL have examined differences between Latinx subgroups, despite the glaring variations in health behaviors and disparities in health outcomes.

5. Conclusions

This is the first study to examine health risk behavior clustering exclusively among Latino men. Our findings showed that a large number of both Mexican and Puerto Rican men engaged in multiple health risk behaviors simultaneously, particularly low physical activity and low consumption of fruits and vegetables. In addition, there were notable differences in the most common clusters between Mexican and Puerto Rican men. Specifically, low consumption of fruits and vegetables along with low physical activity was more common among Mexican men, while low consumption rates of fruits and vegetables and smoking were more prevalent among Puerto Rican men. These findings have implications for preventing cardiometabolic diseases, as engaging in multiple health behaviors has been shown to be associated with cardiovascular disease, cancer, and all-cause mortality [6]. Moreover, these findings are of paramount importance for the development of effective behavioral change interventions tailored to these two groups. Future research is warranted to examine other health risk behaviors and the influence of social and cultural variables in health risk behaviors.

6. Authors' Note

Parts of this manuscript were presented at the 2017 National Hispanic Medical Association Conference, the 2017 University of Illinois at Chicago Psychiatry Research Extravaganza, the 2018 National Hispanic Medical Association Conference, and the 2018 Medical Organization for Latino Advancement's Latino Health Symposium. The data analyses reported in this publication were part of the first author's MPH project.

The words 'Hispanic', 'Hispanic/Latino', and 'Latinx' are used interchangeably, depending on the study being reviewed. 'Latinx' is used as a general inclusive term to refer to all persons of Hispanic/Latin American background. The term 'Latino' is used for participants in the current study, as that was the term used in recruitment and interviews in the parent study (the Latino Men's Health Initiative).

Author Contributions: Conceptualization, A.A. and L.S.-J.; methodology, A.A. and L.S.-J.; formal analysis, A.A. and A.R.; investigation, A.A., A.R. and L.S.-J.; data curation, A.A., A.R. and L.S.-J.; visualization, A.A., C.E.R., A.R. and L.S.-J. writing—original draft preparation, A.A., C.E.R., A.R. and L.S.-J.; writing—review and editing, A.A., C.E.R., A.R. and L.S.-J.; supervision, L.S.-J.; project administration, L.S.-J.; funding acquisition, A.R. and L.S.-J. All authors have read and agreed to the published version of the manuscript.

Funding: The research and data collection were initially funded by the National Institutes of Health's National Cancer Institute under grant numbers R21CA143636 and R1CA143636-S to Lisa Sanchez-Johnsen. The research reported in this publication was also partially supported by a grant from the National Cancer Institute under grant numbers U54CA202995, U54CA202997, and U54CA203000 to co-authors Lisa Sanchez-Johnsen and Alfred Rademaker. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and the data were collected as part of the overall parent study, which received Institutional Review Board (IRB) approval at the University of Illinois at Chicago (IRB 2011-0187) on

27 June 2011. The overall parent study also received IRB approval from the research review board of the Alivio Medical Center. In addition, the University of Illinois at Chicago served as the IRB of record for Northwestern University (STU00204427). The analyses reported in this manuscript also received IRB approval from the University of Illinois at Chicago.

Informed Consent Statement: Written informed consent was obtained for the parent study from all participants involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We extend our appreciation to all participants in this study, our research staff and trainees, as well as to our community partners in the original parent study. We also extend our appreciation to Irma Rodas, B.A., for her technical assistance in the preparation of this manuscript.

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

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The Impact of a Web-Based Lifestyle Educational Program ('Living Better') Reintervention on Hypertensive Overweight or Obese Patients

Pedro Múzquiz-Barberá [1], Marta Ruiz-Cortés [2], Rocío Herrero [3,4], María Dolores Vara [4,5], Tamara Escrivá-Martínez [4,5], Raquel Carcelén [6], Rosa María Baños [4,5], Enrique Rodilla [6,7,*] and Juan Francisco Lisón [2,4]

1 Department of Nursing and Physiotherapy, Faculty of Health Sciences, University CEU-Cardenal Herrera, CEU Universities, 46115 Valencia, Spain; pedro.muzquizbarbera@uchceu.es
2 Department of Biomedical Sciences, Faculty of Health Sciences, University CEU-Cardenal Herrera, CEU Universities, 46115 Valencia, Spain; marta.ruiz3@alumnos.uchceu.es (M.R.-C.); juanfran@uchceu.es (J.F.L.)
3 Department of Psychology and Sociology, Universidad de Zaragoza, 50009 Teruel, Spain; rocio.herrero@uv.es
4 Centre of Physiopathology of Obesity and Nutrition (CIBERobn), CB06/03/0052, Instituto de Salud Carlos III, 46115 Valencia, Spain; m.dolores.vara@uv.es (M.D.V.); tamara.escriva@uv.es (T.E.-M.); rosa.banos@uv.es (R.M.B.)
5 Polibienestar Research Institute, Universitat de València, 46022 Valencia, Spain
6 Department of Medicine and Surgery, Faculty of Health Sciences, University CEU-Cardenal Herrera, CEU Universities, 46115 Valencia, Spain; raquel.carcelen@uchceu.es
7 Hypertension and Vascular Risk Unit, Hospital Universitario de Sagunto, 46520 Valencia, Spain
* Correspondence: rodilla_enr@gva.es; Tel.: +34-962339300 (ext. 339412)

Abstract: 'Living Better', a self-administered web-based intervention, designed to facilitate lifestyle changes, has already shown positive short- and medium-term health benefits in patients with an obesity–hypertension phenotype. The objectives of this study were: (1) to examine the long-term (3-year) evolution of a group of hypertensive overweight or obese patients who had already followed the 'Living Better' program; (2) to analyze the effects of completing this program a second time (reintervention) during the COVID-19 pandemic. A quasi-experimental design was used. We recruited 29 individuals from the 105 who had participated in our first study. We assessed and compared their systolic and diastolic blood pressure (SBP and DBP), body mass index (BMI), eating behavior, and physical activity (PA) level (reported as METs-min/week), at Time 0 (first intervention follow-up), Time 1 (before the reintervention), and Time 2 (post-reintervention). Our results showed significant improvements between Time 1 and Time 2 in SBP (−4.7 (−8.7 to −0.7); $p = 0.017$), DBP (−3.5 (−6.2 to −0.8); $p = 0.009$), BMI (−0.7 (−1.0 to −0.4); $p < 0.001$), emotional eating (−2.8 (−5.1 to −0.5); $p = 0.012$), external eating (−1.1 (−2.1 to −0.1); $p = 0.039$), and PA (Time 1: 2308 ± 2266; Time 2: 3203 ± 3314; $p = 0.030$, $Z = -2.17$). Statistical analysis showed no significant differences in SPB, DBP, BMI, and eating behavior between Time 0 and Time 1 ($p > 0.24$). Implementation of the 'Living Better' program maintained positive long-term (3-year) health benefits in patients with an obesity–hypertension phenotype. Moreover, a reintervention with this program during the COVID-19 pandemic produced significant improvements in blood pressure, BMI, eating behavior, and PA.

Keywords: internet; eHealth; lifestyle; Mediterranean diet; physical activity; weight loss; obesity; hypertension

Citation: Múzquiz-Barberá, P.; Ruiz-Cortés, M.; Herrero, R.; Vara, M.D.; Escrivá-Martínez, T.; Carcelén, R.; Baños, R.M.; Rodilla, E.; Lisón, J.F. The Impact of a Web-Based Lifestyle Educational Program ('Living Better') Reintervention on Hypertensive Overweight or Obese Patients. *Nutrients* **2022**, *14*, 2235. https://doi.org/10.3390/nu14112235

Academic Editors: Abeer M. Mahmoud and Shane Phillips

Received: 2 May 2022
Accepted: 25 May 2022
Published: 27 May 2022

1. Introduction

The implementation of strategies that effectively promote the prevention and treatment of the obesity–hypertension phenotype is urgently required. These must have the clinical objectives of controlling blood pressure (BP) and body composition (fat loss and muscle

mass gain), improving cardiorespiratory fitness and functional capacity, and reducing polypharmacy [1]. In this sense, the most recent clinical guidelines on hypertension (HTN) and obesity (OB) [2–4] agree that promoting a healthy lifestyle should be the first step considered in obese patients with HTN. To achieve these changes, the process must be based on two fundamental pillars: regular physical activity (PA) and healthy eating behavior [5].

PA has been defined as "any bodily movement produced by skeletal muscles that results in energy expenditure" [6]. Specifically, exercise is described as "a subset of physical activity that is planned, structured, and repetitive and has as a final or an immediate objective the improvement of maintenance of physical fitness" [6]. At present, exercise is considered a polypill for the prevention and treatment of numerous health conditions, including chronic diseases such as OB and HTN [5]. Thus, it has been shown that regularly engaging in sustained PA over time is essential to maintain long-term weight loss [1,7]. However, even though moderate weight reductions (~1–3 kg) can be achieved with exercise programs without dietary modifications, the combination of regular PA and healthy eating behaviors, including a decrease in caloric intake [8], is the most effective strategy to address weight loss and its maintenance [1]. Of note, one of the most successful dietary interventions described in the academic literature was from the Prevención con Dieta Mediterránea (PREDIMED) study, and was precisely administered in a Spanish population at high risk for cardiovascular events [9].

The recent academic literature indicates that there was a dramatic decrease in PA during the COVID-19 pandemic, which was especially worrisome in patients with associated metabolic conditions [10]. In hypertensive older adults, unhealthy changes manifested as a reduction in PA and increased sedentary behavior [11]. Other research suggested that unhealthy eating patterns intensified among high-risk patient groups during the pandemic [12]. Similarly, a related study showed that obese individuals spent less time engaging in PA, exercised less intensely, and were more anxious about eating during the pandemic, all of which can make body weight control more difficult [13].

In this context, online interventions can reach different populations, overcoming barriers and limitations, and they represent effective strategies for the prevention and/or treatment of multiple health conditions. However, to date, only four studies [14–17] have analyzed the effectiveness of such treatments in patients with both health conditions—that is, in individuals presenting an OB-HTN phenotype. Of note, none of the four studies performed in this specific area, and only one in non-hypertensive obese adults [18], followed up with patients who had completed an online educational intervention for at least 3 years. In addition, to the best of our knowledge, no research has yet analyzed the effects of a second intervention (reintervention) with an online intervention program in patients with OB, HTN, or any other type of cardiovascular disease.

Given all the above, in this current study, we set out to (1) understand the evolution at 3 years of a group of hypertensive overweight or obese individuals who had followed the 'Living Better' web-based program in 2018 [17]; (2) analyze the effects of completing this program a second time (reintervention) during the COVID-19 pandemic—3 years after the initial intervention—in terms of systolic and diastolic blood pressure (SBP and DBP), body mass index (BMI), number of antihypertensive drugs used, PA, eating behavior, and adherence to the Mediterranean diet.

2. Materials and Methods

2.1. Study Design

This was a prospective quasi-experimental study (ClinicalTrials.gov: NCT04571450). This research was approved by the Human Research Ethics Committee at the Hospital Universitario de Sagunto and followed the ethical guidelines established in the Declaration of Helsinki. The study describes the 3-year follow-up of 29 patients who already received an intervention with the 'Living Better' program in 2018 (n = 105) [17], as well as the result of completing the same program for a second time (reintervention) with the aim of helping to minimize the negative impact of the COVID-19 pandemic on lifestyle. To analyze the

long-term effects of the program, we used the follow-up values obtained at the end of the first study as our starting point (Time 0) [17], which was carried out 9 months after this intervention. We also used the different variables analyzed in 2018 and recorded shortly before the start of the second intervention (Time 1), 21 months later than Time 0. Once this evaluation was completed, the participants started the 3-month online reintervention with the 'Living Better' program. Finally, in order to understand the impact of this second intervention on the health of the participants, all the variables were recorded again at the end of the program (Time 2) (Figure 1). Of note, all the patients were assessed within 3 days at the different study timepoints.

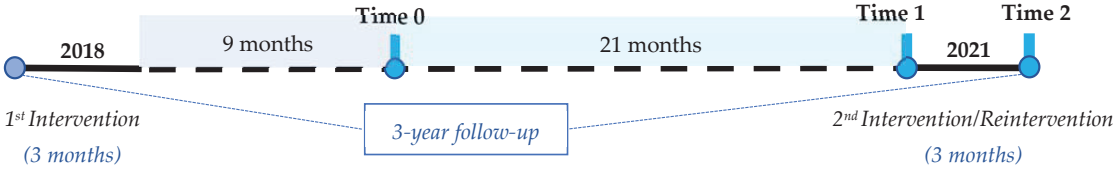

Figure 1. Measurements at trial profile (Time 0, Time 1, and Time 2). Figure 1 shows the time periods when assessments and reintervention were carried out, along the 3-year follow-up.

2.2. Eligibility Criteria

Because this was a reintervention study, the first inclusion criterion was that the patients had participated in the 'Living Better' online program in 2018. In addition, we used the same inclusion and exclusion criteria that we applied in the first study [17]: overweight (BMI between 24.9 kg/m^2 and 30 kg/m^2) or type I obese (29.9 kg/m^2 < BMI < 35 kg/m^2) adults aged 18 to 65 years with HTN. HTN was defined as SBP $\geq$ 140 mmHg or DBP $\geq$ 90 mmHg, or patients who take antihypertensive drugs. We also used the same exclusion criteria: a diagnosis of diabetes, previous ischemic heart or cerebrovascular disease, serious psychiatric disorders, use of more than three antihypertensive medicines, physical impairments that could hinder engagement in PA, receiving other treatments for weight loss, or no access to the Internet.

2.3. Procedure

This study was carried out in the Hypertension and Vascular Risk Unit at the Hospital Universitario de Sagunto (Valencia, Spain) from January 2018 to January 2021 (reintervention period from October 2020 to January 2021). We used the hospital postal service to contact the 105 participants who took part in the 2018 study. Of those who agreed to participate again, the final sample in this work comprised a total of 29 participants (Figure 2). After obtaining their informed consent for participation in the study and formalizing their registration, we incorporated the participants into a single experimental group, which received a 3-month reintervention via the 'Living Better' web-based platform. Furthermore, we telephoned all these individuals to remind them of the program details and to resolve any questions they had.

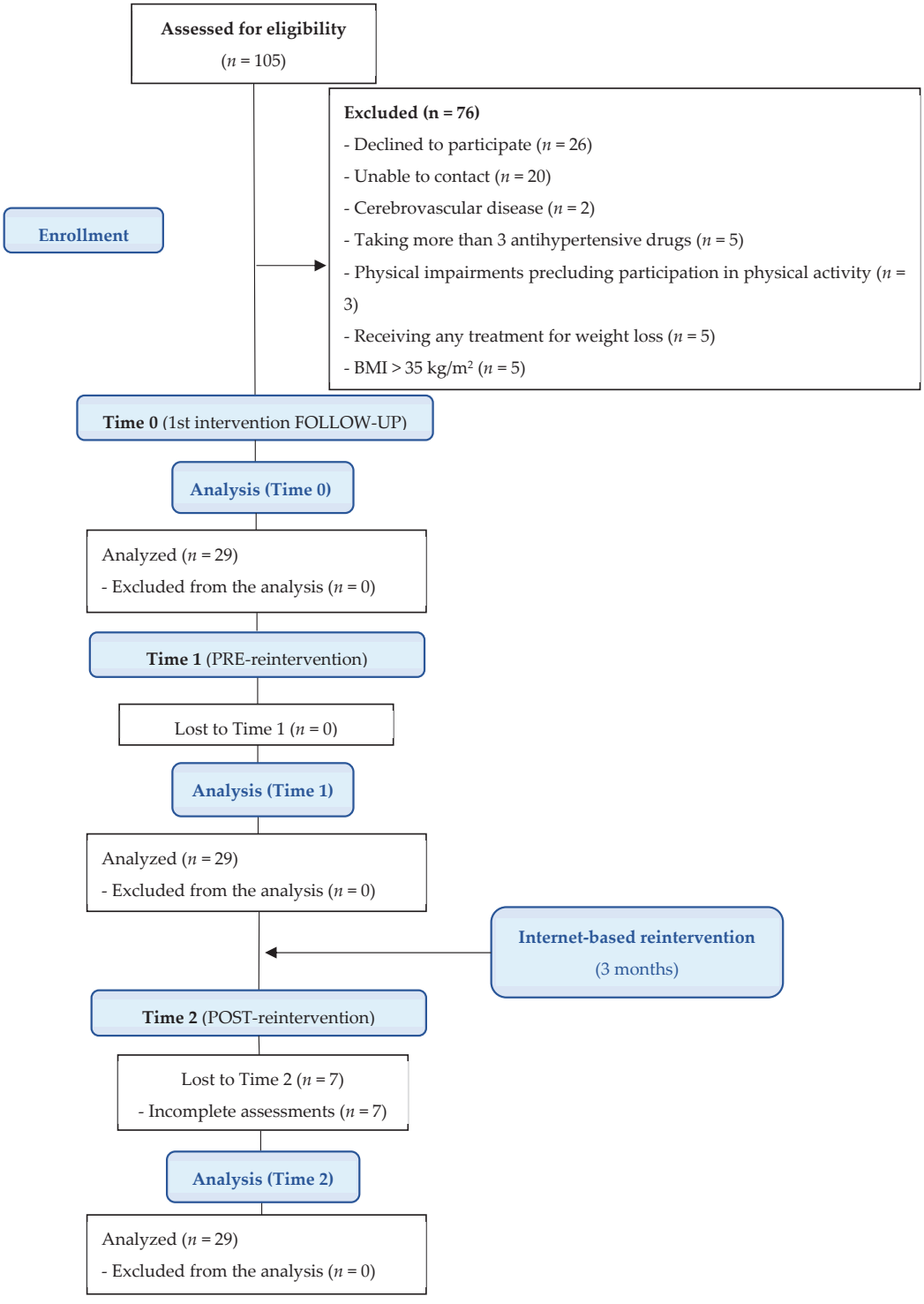

Figure 2. Progression of the participants through the study.

2.4. Intervention

We used the same 'Living Better' program, implemented via the Internet, as in the 2018 study [16,17]. This multimedia, interactive, and self-administered program comprises 9 intervention modules that try to gradually change the eating behavior and PA patterns of the participants. All the modules include videos, texts, tasks, daily records, and files that the patient can download to work on the content. Considering the suggestions of the participants after the first intervention, on this occasion, we had converted part of the written content into audiovisual materials to help facilitate usability. However, despite these changes, the program content was identical to that of the first intervention. More details about the first intervention can be found in Mensorio et al. [16], Lisón et al. [17], Baños et al. [19], and also in the Supplementary Materials (Figure S1 and Video S1).

2.5. Outcome Measures

2.5.1. Systolic and Diastolic Blood Pressure

To avoid coronavirus infections, the health authorities and hospital regulations prohibited access to medical facilities for patients who did not need urgent healthcare. Therefore, the participants were asked to visit a pharmacy close to their home so that the same person (a pharmacist or pharmacy assistant) could record these variables at Time 1 and Time 2 using the same approved device. As in the first intervention, the participants were instructed to record measurements between 8 a.m. and 12:00 p.m. noon to minimize variability in their daytime BP figures. BP was strictly analyzed according to the European Society of Hypertension (ESH)/European Society of Cardiology guidelines and the American College of Cardiology/American Society of Hypertension [2,3], so measurements were performed in the sitting position, in a quiet environment for 5 min before BP measurements, avoiding prior consumption of alcohol or smoking, drinking caffeine, or engaging in strenuous exercise. Three BP measurements were recorded, 1 minute apart, and BP was calculated as the average of the last two BP readings. Additional measurements were performed if the first two readings differed by more than 10 mmHg. Of note, the participants of this study—as with, in general, every patient treated in the Hypertension Unit at the Hospital Universitario de Sagunto—were routinely trained to correctly measure BP in this way.

2.5.2. Weight, Height, and BMI

Because of the aforementioned COVID-19-related health concerns, these variables were also recorded in local pharmacies, following the same indications. Specifically, clothing was standardized during weight measurement, and patients were instructed to visit the pharmacy while fasting and preferably always at the same time to avoid the possibility that any food or drink ingested could influence their data. BMI was calculated by dividing patient weight by their height squared (kg/m^2).

2.5.3. Antihypertensive Drugs

The patient registered the number of antihypertensive drugs they used through the intervention program platform.

2.5.4. Physical Activity Levels

The short version of the International Physical Activity Questionnaire (IPAQ-SF) was used [20,21] to assess the time that each subject had spent being active in the 7 days prior to completion of the survey. Different scores are awarded in the IPAQ-Short, depending on the time spent engaging in moderate or vigorous activities, walking, or sitting each week. The unit of measurement for this questionnaire is METs-min/week, which expresses the average of each individual's metabolic expenditure per minute while engaging in weekly PA. Thus, higher figures reflect a higher level of activity, while lower values express a lower level of weekly PA [20,21]. Data should be interpreted using the formula published by Ainsworth et al. [22] to classify their PA levels as high (>1500 METs-min/week), moderate (600–1500 METs-min/week), or low (<600 METs-min/week).

2.5.5. Eating Behavior

To analyze the eating behavior of the patients, we employed the 'Dutch Eating Behavior Questionnaire' (DEBQ) [23,24], which comprises 33 items and uses a 5-point Likert scale to evaluate 3 eating styles, emotional eating (13 items), external eating (10 items), and restrained eating (10 items), with higher scores indicating greater agreement with the eating behavior statements.

2.5.6. Adherence to the Mediterranean Diet

Eating habits were recorded before (Time 1) and after (Time 2) the reintervention using the 'Mediterranean Diet Adherence Screener' (MEDAS) from the PREDIMED study [25]. This questionnaire assesses adherence to the Mediterranean diet through 14 items, 12 of which are related to the frequency of food consumption, while 2 are about dietary habits linked to the Mediterranean diet. Each item is scored with a value of 0 or 1 and, based on the final score, the patients were classified as having low (0–5 points), medium (6–9 points), or high ($\geq$10 points) adherence to the Mediterranean diet.

2.5.7. Satisfaction with the Reintervention

As in the first study [17], this variable was evaluated on a scale from 0 (minimum satisfaction) to 10 (maximum satisfaction).

2.5.8. Adherence to Reintervention

This was analyzed through the data registered by the participants on the platform. This also allowed us to gauge the degree of completion of the different program modules by each participant—in other words, how many of the nine modules they had reviewed.

2.6. Statistical Analysis

The statistical analyses were performed according to the intention-to-treat paradigm using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) for Windows, and the statistical significance was set at $p < 0.05$ for all our analyses. The data in this study are presented as mean (SD). Compliance with the assumption of normality was checked for each dependent variable using the Shapiro–Wilk test. One-way ANOVA tests followed by Bonferroni post-hoc tests were performed for the variables that met the assumption of normality (SBP, DBP, BMI, and eating behavior). The effect sizes were estimated using the ηp^2 and were interpreted following Cohen's guidelines for small, moderate, and large effect sizes (ηp^2 = 0.01, 0.06, or 0.14, respectively). Friedman tests followed by non-parametric Wilcoxon tests to compare the three study timepoints (Time 0, Time 1, and Time 2) were used for the variables that violated the assumption of normality (PA and antihypertensive drugs). In addition, *t*-tests for related samples were performed to compare the level of adherence to the Mediterranean diet before and after the reintervention (Time 1 vs. Time 2), as well as to contrast the degree of participant satisfaction after the reintervention compared with the first intervention. Adherence to the reintervention was estimated by calculating the average percentage of the 9 'Living Better' program modules completed by the 29 participants. Finally, at Time 0, depending on whether the assumption of normality was fulfilled, *t*-tests (for independent samples) or Mann–Whitney U tests were carried out for the different study variables to compare the 29 reintervention participants to the 76 participants excluded from this study.

3. Results

3.1. Reported Changes in the SBP, DBP, BMI, and Eating Behavior

Table 1 shows the patient values for the variables prior to the second intervention (Time 1). Specifically, regarding BMI, 62% of the participants (18 of 29) were overweight at Time 1, while the other 11 patients (38%) had type I obesity. In addition, Table 2 shows data reported from the different timepoints and the results of the post-hoc ANOVA analysis. As shown, there were significant differences between the start of the second intervention (Time 1)

and the end of the program (Time 2) in all variables—except for restrained eating—with statistically significant improvements and large effect sizes ($\eta p^2 > 0.21$) after completing the reintervention. However, the statistical analysis did not show significant differences between the end of the first intervention (Time 0) and the beginning of the second one (Time 1) for any of these four variables ($p > 0.24$).

Table 1. Participant characteristics.

VARIABLES		Time 1; Mean (SD) [a]
Sex (*n*)	Women	8
	Men	21
Age (years)		57.3 (10.0)
Systolic blood pressure (mmHg)		129.6 (12.2)
Diastolic blood pressure (mmHg)		78.6 (8.1)
Weight (kg)		84.1 (11.0)
BMI (kg/m^2)		29.2 (2.4)
Antihypertensive drugs (*n*)		1.6 (1.4)
Physical activity level (METs-min/week)		2308 (2266)
Eating behavior (points)	Emotional eating	27.1 (10.7)
	External eating	28.4 (6.6)
	Restrained eating	27.0 (6.0)
Adherence to the Mediterranean diet (points)		8.2 (2.1)

[a] Time 1 (average values prior to patient reintervention).

3.2. Differences Found in Antihypertensive Drugs and PA

All patients in our study were receiving antihypertensive treatment. The Friedman test did not indicate any statistically significant changes in the number of antihypertensive drugs used between the different evaluation points (Time 0: 1.7 ± 1.2; Time 1: 1.6 ± 1.4; and Time 2: 1.6 ± 1.4; $p = 0.439$), although there were significant differences for PA (Time 0: 4024 ± 3676; Time 1: 2308 ± 2266; and Time 2: 3203 ± 3314; $p = 0.005$). Specifically, the results of the Wilcoxon tests showed differences between Times 0 and 1 ($p = 0.015$, $Z = -2.43$) and Times 1 and 2 ($p = 0.030$, $Z = -2.17$).

3.3. Results Analyses of Adherence to the Mediterranean Diet, Satisfaction, and Adherence to the Reintervention

Regarding adherence to the Mediterranean diet, *t*-tests showed that there were no statistically significant differences ($p = 0.100$) between the time before (Time 1: 8.2 ± 2.1) and immediately after the reintervention (Time 2: 8.8 ± 1.7). Furthermore, participants reported a higher level of satisfaction with the program after the second intervention compared to the first one, although these findings did not reach statistical significance (first intervention: 6.8 ± 2.3, second intervention: 8.0 ± 1.4; $p = 0.080$). With regard to adherence to the reintervention, seven patients withdrew before completing the first module, 66% of the 29 participants had looked at more than half of the program (at least 5 of the 9 modules), and 38% had completed all of it. Finally, at Time 0, the comparison between the 29 volunteers who agreed to participate in the reintervention and the 76 participants excluded from the study showed no statistically significant differences in any of the studied variables ($p > 0.29$), except for the BMI, which was higher in the excluded patient group (29.0 ± 2.5 and 30.2 ± 2.8, respectively; $p = 0.033$). However, a subsequent analysis verified that the differences had already existed before the first intervention between these two groups (29.3 ± 2.6 versus 30.5 ± 2.6; $p = 0.033$).

Table 2. Comparisons for Time 0 versus Time 1 versus Time 2.

VARIABLES		Baseline [a]	Time 0 [b]	Time 1 [c]	Time 2 [d]	Time 0 vs. Time 1		Time 1 vs. Time 2	
						Difference [e] (95% CI)	*p*	Difference [f] (95% CI)	*p*
Systolic blood pressure (mmHg)		128.8 (11.5)	127.3 (12.7)	129.6 (12.2)	124.9 (11.1)	2.3 (−4.0 to 8.5)	1.000	−4.7 (−8.7 to −0.7)	0.017 *
Diastolic blood pressure (mmHg)		77.0 (6.6)	76.4 (6.7)	78.6 (8.1)	75.1 (8.9)	2.2 (−2.0 to 6.4)	0.600	−3.5 (−6.2 to −0.8)	0.009 **
BMI (kg/m^2)		29.3 (2.6)	28.9 (2.5)	29.2 (2.4)	28.6 (2.3)	0.3 (−0.4 to 1.0)	0.895	−0.7 (−1.0 to −0.4)	<0.001 **
Eating behavior (points)	Emotional eating	28.8 (10.6)	27.8 (8.6)	27.1 (10.7)	24.3 (9.0)	−0.8 (−3.7 to 2.2)	1.000	−2.8 (−5.1 to −0.5)	0.012 *
	External eating	30.6 (6.1)	29.5 (6.4)	28.4 (6.6)	27.3 (7.0)	−1.1 (−3.3 to 1.1)	0.640	−1.1 (−2.1 to −0.1)	0.039 *
	Restrained eating	27.9 (6.6)	28.6 (6.6)	27.0 (6.0)	26.9 (6.0)	−1.6 (−3.9 to 0.7)	0.248	−0.2 (−1.6 to 1.2)	1.000

[a] Baseline: average values obtained prior to the first intervention, presented as mean (SD). [b] Time 0: average values obtained at the end of the first study, presented as mean (SD). [c] Time 1: average values prior to patient reintervention, presented as mean (SD). [d] Time 2: average values post patient reintervention, presented as mean (SD). [e] Difference was calculated as Time 1 (PRE-reintervention) minus Time 0 (1st intervention FOLLOW-UP). [f] Difference was calculated as Time 2 (POST-reintervention) minus Time 1 (PRE-reintervention). * $p \leq 0.05$; ** $p \leq 0.01$.

4. Discussion

This study indicates that the 29 hypertensive overweight or obese patients enrolled in the reintervention had maintained long-term benefits in terms of reduced BMI and BP at a 3-year follow-up after having completed the 'Living Better' online intervention [16,17]. Likewise, our results show that these variables significantly improved after the same group of patients repeated the program a second time (reintervention). To the best of our knowledge, this is the first work using a web-based program aimed at promoting a healthy lifestyle based on psychoeducation, regular engagement in PA, and the establishment of healthy eating behavior with such a long-term follow-up time. It is also the first study to describe the effects of a reintervention in patients with an OB-HTN phenotype.

Our results did not show any significant changes in any of the study variables (SBP, DBP, BMI, antihypertensive drugs, or eating behavior) at the 3-year follow-up, compared to the first intervention in 2018, with the exception of the level of PA, which had significantly worsened. This decline may have been because of the restrictions to movements and access to sports spaces imposed by governmental authorities as a result of the COVID-19 pandemic at the time of this work. In this sense, recent research indicates that there was a significant decrease in PA at this time, accompanied by an increase in sedentary habits, due to these restrictions [10,11]. Also of note, the eating behavior of the study patients did not significantly worsen during that time. Indeed, the 'Living Better' program has already been shown to effectively improve emotional eating and other psychological variables related to eating and quality of life (anxiety and stress) [16]. These results are consistent with the absence of significant changes in BP and BMI, together indicating the long-term effectiveness of the 'Living Better' program.

To help deal with the possible negative lifestyle effects of the COVID-19 pandemic on patients with the OB-HTN phenotype (for example, decreased PA), we decided to implement a second intervention with the same program. Given the self-administered, interactive, multimedia, and web-based nature of the platform, we hypothesized that repeating this program could reinforce and enhance the knowledge that the patients had acquired after the first intervention, helping them to face the barriers and thereby perhaps minimizing the negative impact of the situation on their lifestyle and health.

The results that we obtained after administering the reintervention confirmed our hypothesis. Thus, despite the restrictions imposed by the pandemic, the participants had significantly increased their levels of PA—after 3 months of reintervention—by approximately 30%, or around 900 METs-min/week. In addition to the improvements in PA, as already demonstrated in the first intervention in 2018 [16], reintervention with the 'Living Better' program also positively influenced emotional eating and external eating. In fact, one of the goals of this program is to change eating behavior (generating a more conscious and less impulsive eating style) by using psychoeducation, eating tricks, and self-control strategies. This finding is relevant because eating styles are considered to be multi-dimensional, stable, and related to OB [26]. The latter is important in the context of the negative emotions such as anxiety and panic generated by the COVID-19 pandemic, which have been associated with unhealthy eating behavior in populations with higher rates of OB [27,28]. Furthermore, adherence to the Mediterranean diet before reintervention was close to the upper limit of the 'medium adherence' range (8.2 points on the MEDAS questionnaire) [25], perhaps because of the effect of the first intervention. Nonetheless, the reintervention still produced a slight increase in the score by 0.6 points.

Therefore, presumably as a consequence of improvements in PA and eating behavior after the reintervention, the participants had reduced their body weight by an average of 2 kg, which translated into a significant reduction in BMI by 0.7 kg/m^2. Of special note, this BMI reduction was even higher than that achieved after the first intervention in 2018 (0.4 kg/m^2) [17]. In addition, the literature also reflects the direct impact that weight loss has on BP values [29]. In this sense, compared to our first study [17], the SBP and DBP of the reintervened patients also decreased further, possibly as a consequence of the greater BMI reduction. In these patients, SBP and DBP decreased by 4.7 and 3.5 mmHg, respectively

($p = 0.017$ and $p = 0.009$), compared to the non-significant reduction in SBP (−2.6 mmHg, $p = 0.15$) and the lower reduction in DBP (−2.2 mmHg, $p = 0.05$) that we reported after the first intervention in 2018. These post-reintervention improvements also exceeded those reported in the meta-analysis by Liu et al. on Internet-based lifestyle counselling [30], in which SBP and DBP were reduced by a mean of 3.8 mmHg and 2.1 mmHg, respectively. Likewise, it is important to note that the improvements that we found in this research were not the result of a change in medication, because no significant differences were reported by the participants at any of the timepoints examined in the number of antihypertensive drugs used.

In terms of program engagement [31], the percentage of participants who completed our entire program was lower (38%) than in our first intervention [17] or similar e-counselling lifestyle interventions [32]. The low completion rate for the whole program during the reintervention may have been partly because of the limitations caused by the COVID-19 pandemic, perhaps forcing the population to adapt their working hours and spaces, as well as reducing the availability of personal time and resources [33,34]. This phenomenon may also have been because the participants had remembered some of the educational content from the first intervention, leading them to complete only the modules that they considered necessary. Indeed, two thirds of the participants completed at least half of the 'Living Better' program (five or more modules). Moreover, the mean participant satisfaction with the reintervention was 1.2 points (out of 10) higher than the average from the first study [17], although this did not reach statistical significance. This difference may be because of the alterations we made to the program presentation by including more audiovisual content [35,36], as suggested by the patients after the first intervention.

At this point, it is important to highlight that the Internet has been shown as an effective means to promote healthy lifestyles in order to help prevent and treat chronic diseases. This is because it can reach more people (including those with limited access to health services or low levels of social support) and it can provide patients with more intensive contact with clinicians at a lower economic cost than conventional face-to-face programs [37,38]. Additionally, Internet-based platforms can provide immediate, easily accessible, individually tailored (one-on-one), and permanent (accessible at any time) support to patients in the comfort of their own homes. All these advantages were especially relevant in the context of the COVID-19 pandemic, which was ongoing while this study was implemented. Therefore, the long-term effects of the web-based 'Living Better' program and those obtained after a reintervention with the same program were remarkable and should be scientifically valued. They minimized the profound negative impact of COVID-19 on the health of these patients—who all had an OB-HTN phenotype—and even managed to improve their health profiles.

Limitations

The main limitation of this study was the absence of a control group. Of note, since the sample size was small—because the study design was a continuation of previous work—and mostly for ethical reasons, all 29 participants were assigned to a single experimental group so that this population, which was especially vulnerable to COVID-19, received effective treatment during this trial. Although the positive eating behavior, PA, BMI, and BP results were similar to those obtained in our previous 'Living Better' randomized controlled trial, the absence of a control group must be considered when interpreting the effects of this reintervention. In addition, although, prior to reintervention, we were unable to identify any differences in the variables in the 29 participants and the 76 patients excluded from the study, we cannot rule out the possibility of a selection bias. Therefore, these findings should be interpreted with caution. Finally, the participants were unable to go to the hospital for BMI and BP measurements before and after the reintervention because of the COVID-19 pandemic restrictions. However, this problem was mitigated by having these measurements completed by the same person (a pharmacist or pharmacy assistant) using

the same approved devices both times, and strictly following the ESH protocol, as in the first intervention.

5. Conclusions

This study shows that the 'Living Better' web-based program had long-term (3-year) benefits for the health of patients with an obesity–hypertension phenotype. In addition, given the context of the COVID-19 pandemic, we evaluated the effects of implementing a second intervention in these patients with the same program to try to reduce the potential negative consequences on their lifestyles. The reintervention showed significant improvements, for the second time, in eating behavior, physical activity levels, BMI, and blood pressure.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14112235/s1, Figure S1: 'Living Better' program modules; Video S1: Video summary of the 'Living Better' web-based program.

Author Contributions: P.M.-B., R.M.B., E.R. and J.F.L. conceived this research methodology and wrote/prepared the original draft. M.R.-C. and R.H. were responsible for the methodology. P.M.-B. and J.F.L. conducted a formal analysis. R.M.B., E.R. and J.F.L. managed the investigation. P.M.-B., M.R.-C. and R.H. reviewed and edited the manuscript. R.C., M.D.V. and T.E.-M. were responsible for visualization. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study, where human participants were involved, was reviewed and approved on 6 June 2019 by the University CEU-Cardenal Herrera Ethics Committee (CEI19/085). This research was also approved by the Human Research Ethics Committee at the Hospital Universitario de Sagunto and followed the ethical guidelines established in the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

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

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: This work was supported by grants from the Generalitat Valenciana (Subvenciones para grupos de investigación consolidables—AICO/2019/331) and from the University CEU-Cardenal Herrera (Convocatoria de Consolidación de Indicadores CEU-UCH 2021-2022/INDI21/31). The Centre of Physiopathology of Obesity and Nutrition (CIBERobn) is an initiative of Carlos III Health Institute. Author P.M.-B. is grateful to University CEU-Cardenal Herrera (CEU Universities, Valencia, Spain) for the doctoral scholarship.

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

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The Association of Dietary Intake, Oral Health, and Blood Pressure in Older Adults: A Cross-Sectional Observational Study

Pinta Marito [1,2], Yoko Hasegawa [1,3,*], Kayoko Tamaki [4], Ma Therese Sta. Maria [1,5], Tasuku Yoshimoto [1], Hiroshi Kusunoki [4], Shotaro Tsuji [6], Yosuke Wada [7], Takahiro Ono [1], Takashi Sawada [8], Hiromitsu Kishimoto [3] and Ken Shinmura [4,*]

1 Division of Comprehensive Prosthodontics, Faculty of Dentistry & Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8514, Japan; pintamarito@dent.niigata-u.ac.jp (P.M.); mari18@dent.niigata-u.ac.jp (M.T.S.M.); yoshimoto@dent.niigata-u.ac.jp (T.Y.); ono@dent.niigata-u.ac.jp (T.O.)
2 Department of Prosthodontics, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia
3 Department of Dentistry and Oral Surgery, Hyogo College of Medicine, Nishinomiya 663-8501, Japan; kisihiro@hyo-med.ac.jp
4 Department of General Internal Medicine, Hyogo College of Medicine, Nishinomiya 663-8501, Japan; kayoko_tamaki@hotmail.com (K.T.); kusunoki1019@yahoo.co.jp (H.K.)
5 Department of Prosthodontics, College of Dentistry, Manila Central University, Caloocan 1400, Philippines
6 Department of Orthopaedic Surgery, Hyogo College of Medicine, Nishinomiya 663-8501, Japan; tj13041305sho@gmail.com
7 Department of Rehabilitation Medicine, Hyogo College of Medicine Sasayama Medical Center, Sasayama 669-2321, Japan; yu-wada@hyo-med.ac.jp
8 Hyogo Dental Association, Kobe 650-0003, Japan; sawada@fc.hda.or.jp
* Correspondence: cem17150@dent.niigata-u.ac.jp (Y.H.); ke-shimmura@hyo-med.ac.jp (K.S.); Tel.: +81-25-227-2891 (Y.H.); +81-798-45-6865 (K.S.)

Citation: Marito, P.; Hasegawa, Y.; Tamaki, K.; Sta. Maria, M.T.; Yoshimoto, T.; Kusunoki, H.; Tsuji, S.; Wada, Y.; Ono, T.; Sawada, T.; et al. The Association of Dietary Intake, Oral Health, and Blood Pressure in Older Adults: A Cross-Sectional Observational Study. *Nutrients* **2022**, *14*, 1279. https://doi.org/10.3390/nu14061279

Academic Editors: Abeer M. Mahmoud and Shane Phillips

Received: 4 February 2022
Accepted: 14 March 2022
Published: 17 March 2022

Abstract: Hypertension is related to impaired mastication that causes malnutrition, declining the general health of older adults. This study assessed the role of dietary intake in the relationship between oral health and blood pressure. Eight hundred ninety-four adults aged ≥65 years who independently lived in rural regions of Japan participated in this study. Hypertension was classified according to the guidelines of the Japanese Society of Hypertension. The oral condition was evaluated by analyzing the remaining teeth, occlusal force, posterior occlusal support, masticatory performance, oral moisture, and oral bacterial level. Dietary intake was assessed using a brief self-administered dietary history questionnaire. Mann-Whitney U, chi-square, Kruskal-Wallis tests, and logistic regression analyses were used to elucidate the factors related to hypertension. Normotensive, hypertensive, and history of hypertension were observed in 30.9%, 23.8%, and 45.3% of the participants, respectively. The factors significantly associated with the hypertension were age, body mass index, posterior occlusal support condition, and sodium-to-potassium ratio related to salt intake and/or vegetable intake. Participants without posterior occlusion significantly had higher risk of hypertension (odds ratio = 1.72). This study suggested that there was an association between oral health and hypertension, while the loss of occlusal support may influence nutritional intake conditions.

Keywords: hypertension; blood pressure; oral health; dietary intake; brief-type self-administered diet history questionnaire

1. Introduction

The prevalence of hypertension has more than doubled in the last 30 years, making it one of the leading causes of disease and mortality worldwide, with an estimated 1.28 billion people suffering from hypertension in 2019 [1,2]. Hypertension has become one of the leading causes of death in Japan among noncommunicable diseases [3,4]. Alarmingly, the number of people with hypertension in Japan is estimated to be 43 million [1,5], but only

50% received treatment, while 25% had controlled blood pressure (BP) [5]. Furthermore, approximately 70% of older adult population (≥75 years) of Japan has hypertension [6]. According to the Japan Society of Hypertension, individuals who are ≥ 75 years old with a BP ≥ 140/90 mmHg are considered hypertensive [5]. Hypertension is a complex medical condition caused by several factors. Previous studies have identified that periodontal disease [7], occlusal status [8], and tooth loss [8–10] are associated with hypertension. However, the role of oral health in hypertension is yet to be clarified. Identifying the risk factors for hypertension, even those with marginal risk, is crucial to devise strategies to prevent the development of hypertension and thus prevent cardiovascular disease. Several studies have reported that hypertension is associated with oral health, including impaired mastication, poor oral hygiene, and oral inflammation [7,9–11].

Oral health, which is an indicator of general health, can be affected by a range of diseases and conditions that include dental caries, periodontal disease, and tooth loss [12]. Teeth and oral function constitute the main pathways considered vital in connecting oral to general health. According to the Health 21 plan of Japan, improvement of oral function is the primary target for older adults [13], and the number of teeth has been long established as one of the indicators of the oral health condition [14]. According to the Japan Dental Diseases Survey in 2016, approximately 280,000 and 100,000 patients were estimated to have minor (1–8 missing teeth in one jaw) and major (9–14 missing teeth in one jaw) tooth loss, respectively [15]. Further, adults (≥75 years) lose a minimum of ten teeth per year [16]. A study has reported that tooth loss, particularly in older adults, was associated with malnutrition [17]. Tooth loss invariably leads to the decline of mastication ability, changes in food selection and dietary intake, and changes in nutrient intake, all of which, consequently, have an adverse effect on general health, increasing the risk of systemic diseases, frailty, and mortality [17–22].

As oral and general health decline with aging and disease(s) [20,21,23–26], the number of unchewable food particles increases over time, leading to changes in food selection and eating habits [19,23,24]. According to the 2018 National Health Nutrition Survey in Japan, approximately 25% of people (≥60 years of age) reported a decline in masticatory function, which implies that they were unable to chew a variety of food [13]. Mastication is the first step of the digestive process of breaking food into smaller particles for swallowing that allows more nutrient absorption, which is essential for the maintenance of health, especially in older adults [24,27]. Numerous studies have reported that masticatory function is influenced by several factors, such as the number of remaining teeth [21,28], posterior occlusal contact [21,29,30], occlusal force [21,30], salivary secretion [28,30], and tongue function [28,31]. Tooth loss influences an individual's food choice and dietary intake, leading to maladaptive behaviors. For example, an individual with tooth loss will prefer eating soft and easy-to-chew foods [32], and will avoid fiber-rich and nutrient-dense foods, such as raw fruits and vegetables, nuts, meats, and grain products, thereby increasing fat, sugar, other carbohydrates, and processed food consumption [32,33].

To measure an individual's usual food consumption and dietary intake, a food frequency questionnaire can be utilized. Food questionnaires have become one of the main research tools in nutritional epidemiology [34]. In Japan, Sasaki et al. [35] developed a brief self-administered diet history questionnaire (BDHQ) to assess the Japanese diet, which uses food frequency and dietary history. The BDHQ is a validated food frequency questionnaire that estimates the dietary intake of 58 food and beverage items during the preceding month. It consists of the following five sections: intake frequency of food and non-alcoholic beverages, daily intake of rice and miso soup, frequency of drinking alcoholic beverages and amount per drink for five alcoholic beverages, usual cooking methods, and general dietary behaviors [35–37]. The food and beverage items included in the questionnaire were mainly from a food list used in Japan's National Health and Nutrition Survey, which is based on foods commonly consumed in Japan [35].

It is widely established that there are relationships between nutrition and hypertension [38–40], and between oral health and hypertension [7–10,41]. A study by Fushida

et al. [41] elucidated the link between high BP and decreased masticatory performance; however, their study did not assess the role of oral health in nutrition. As stated by the authors, a non-direct causal relationship was assumed between high BP and decreased masticatory ability [41]. Nutritional status is expected to be very strongly associated with the relationship between high BP and decreased masticatory performance, among several other expected confounding factors [8,41]. Therefore, the authors felt the need to investigate further the association of oral health with hypertension, indirectly, by assessing nutrition, to better understand the cardiovascular demographics in older adults.

The authors hypothesized that impaired oral health can cause nutritional imbalances, which might affect blood pressure. Hence, in this study, we aimed to investigate the dietary intake of the Japanese older adult population to clarify the role of oral health in nutrition and hypertension.

2. Materials and Methods

2.1. Study Design and Research Subjects

This prospective cross-sectional study was approved by the Institutional Review Board of Hyogo Medical College (approval number: Rinhi 0342), and it was also part of the frail elderly study in the Tamba Sasayama-Area (FESTA), a cohort study conducted in Tamba Sasayama-Area Hyogo Prefecture, Japan. The study population was composed of healthy community-dwelling older adult individuals aged 65 years and above who voluntarily participated and provided written informed consent in a joint medical and dental study between April 2016 and December 2019.

Research participants were recruited through advertising in local newspapers and posters at the Hyogo College of Medicine Sasayama Medical Center. Participants included in this FESTA study were independent older adult who needed less than level 1 care in the long-term care insurance system in Japan. The exclusion criteria were participants suspected of having moderate to severe dementia (Mini-Mental Condition Screening score < 20). Participants were informed on the aim and method of the study before beginning the comprehensive assessments.

We recruited 921 participants (the baseline data) with complete anthropometric and BDHQ data, while we excluded six participants without oral health assessment data. Based on the BDHQ data, we also excluded 21 participants with a calorie intake of <600 kcal/day or >4000 kcal/day (<600 kcal/day is equivalent to half the calorie intake required for the lowest physical activity while >4000 kcal/day is equivalent to 1.5 times the energy intake required for medium physical activity) [42] because extremely low or high calorie intake values were suspected to be an improper response in the BDHQ survey [36]. After excluding the potential outliers, we enrolled 894 participants aged 65 years and above (mean age: 74.3 $\pm$ 5.8 years, 282 men and 612 women) in this study (Figure 1).

2.2. Blood Pressure Assessment

Blood pressure measurement was performed between 10:00 and 13:00. Participants were allowed to rest for 10 min prior to the measurement to avoid recording incorrect data. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice during daytime (with a 1-minute interval per measurement) using a fully automatic calibrated oscillometer (BP-203 RVII, Colin Co., Aichi, Japan) with an upper-arm cuff device (the participant's arm was resting on the table during the measurement). The average of two measurements was considered [5].

According to the criteria of the Japanese Society of Hypertension (JSH) 2019 guidelines for the management of hypertension, participants with SBP < 140 mmHg/DBP < 90 mmHg and SBP $\geq$ 140 mmHg/DBP $\geq$ 90 mmHg or those found using hypertensive medication are classified as normal and hypertensive, respectively [5,6]. Based on this, we grouped the participants as "Normotensive" (SBP of <140 mmHg and DBP < 90 mmHg), "Hypertensive" (SBP of $\geq$140 mmHg and/or DBP $\geq$ 90 mmHg), and "History of hypertension" (history of hypertension in the medical interview and/or is taking hypertension medication). The

medications that the participants in the History of hypertension group were taking are shown in Appendix A Tables A1 and A2.

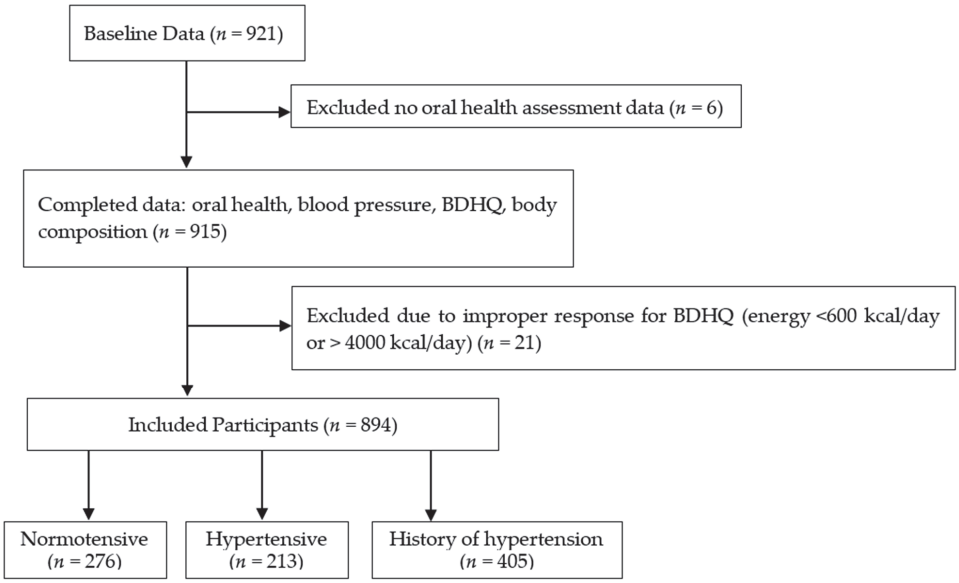

Figure 1. Flow diagram demonstrating the recruitment and group assignment of the study participants. BDHQ; a brief self-administered diet history questionnaire. Normotensive: Participants with systolic blood pressure (SBP) < 140 mmHg and diastolic blood pressure (DBP) < 90 mmHg, Hypertensive: Participants with SBP of ≥140 mmHg and/or DBP ≥ 90 mmHg, History of hypertension: Participants who answered that they had a history of hypertension in the medical interview and/or is taking hypertension medication.

The difference between the SBP and DBP was recorded as the pulse pressure (PP). PP indicates large-artery stiffness, which is a blood pressure indicator for chronic heart disease risk and advanced atherosclerosis [5,43].

2.3. Survey Questionnaire

We asked the participants to complete a survey questionnaire collecting information about their sociodemographic characteristics such as age, sex, education, and living arrangements. Education was defined by the number of years for completion based on the Japanese education system [44], while the living arrangement was defined as alone or with family if more than one family member. Information on health included exercise and smoking habits, and chronic diseases such as diabetes mellitus, dyslipidemia, chronic kidney disease, cardiovascular disease, and stroke were collected from the participants.

2.4. Physical Component Assessments

We determined the participants' body mass index (BMI), skeletal muscle mass, body fat mass, and percent body fat using a body composition analyzer InBody770 (InBody, Tokyo, Japan) with a bio-electrical impedance. The BMI (kg/m^2) was defined as the weight divided by the square of the height. Skeletal muscle mass was the summation of the mass of the upper and lower extremities [45,46], body fat mass was the summation of fat at the surface level and internal fat, and percent body fat, an indicator of the risk of obesity, was body fat mass divided by total weight [47].

2.5. Oral Health Assessment

Assessment of oral health was conducted by calibrated dental examiners. The examinations were performed under bright artificial lighting while the participants sat on a reclining care chair. Oral health was defined on the basis of the number of remaining teeth and oral functions [13,48,49]. In this study, we determined the number of remaining teeth (remaining root fragments and third molars; min, max: 0, 32) [49] and oral functions (maximum occlusal force, posterior occlusal contact, masticatory performance, oral moisture, and oral bacterial counts). To determine the maximum occlusal force, the participants were asked to bite an Occlusal Force-Meter GM10 (Nagano Keiki, Tokyo, Japan). When one of the first molars was missing, the participants were asked to bite on the closest teeth based on the location of the missing first molar [48,50]. Denture-wearing participants were asked to wear their dentures during the measurements. Masticatory performance (MP) assessment was performed using a test gummy jelly (UHA Mikakuto Co., Ltd., Osaka, Japan). Participants were instructed to chew the gummy jelly 30 times and expectorate all the chewed gummy particles on top of a gauze spread over a paper cup. Masticatory performance was evaluated using the visual scoring method on a 10-point scale (0 = minimum to 9 = maximum) [51]. Posterior occlusal contact was defined as the tooth with an occluding antagonist, which may be a natural dentition or a fixed denture [15,21]. According to Eichner's classification, the occlusal contact in each of the premolar and molar regions was classified into group A (occlusal contact in all four occlusal contact zones), group B (occlusal contact in one to three occlusal contacting zones), and group C (no antagonist contacts in the dentition) [15,21]. In this study, the participants were divided into three groups according to the availability of posterior teeth as the posterior occlusal contact; Eichner A1, A2, A3, B1, B2, and B3 (Figure 2A, "with posterior occlusion (w/PO) group"), Eichner B4, C1, and C2 (Figure 2B, "without posterior occlusion (w/o PO) group"), and Eichner C3 (Figure 2C, "edentulous group") [19].

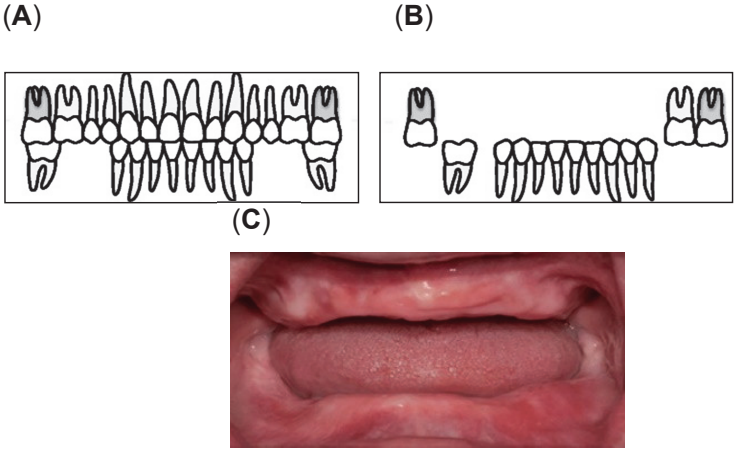

Figure 2. Classification of the Posterior Occlusal Contact: (**A**) With posterior occlusion (with PO) group consisting of Eichner A1, A2, A3, B1, B2, and B3; (**B**) Without Posterior occlusion (without PO) group consisting of Eichner B4, C1, and C2 [52]; (**C**) the edentulous group [53].

Oral moisture was assessed twice by measuring the wetness on the dorsum of the tongue and buccal mucosa using an oral moisture meter (Mucus®, LIFE Co., Ltd., Saitama, Japan). A dielectrophoretic impedance measurement method (Panasonic Healthcare Co., Tokyo, Japan) was used to evaluate the bacterial count on the tongue surface [54,55]. The machine rates bacterial counts from levels 1 to 7 [56]; level 1, 2, 3, 4, 5, 6, and 7 indicate the bacterial counts of $<10^5$, $\geq 10^5$ and $<10^6$, $\geq 10^6$ and $<3.16 \times 10^6$, $\geq 3.16 \times 10^6$ and $<10^7$, $\geq 10^7$ and $<3.16 \times 10^7$, $\geq 3.16 \times 10^7$, $<10^8$, and $\geq 10^8$, respectively [49].

2.6. Nutrition Assessment Methods

Dietary intakes were evaluated using a brief-type self-administered diet history questionnaire (BDHQ), a previously validated fixed-portion type food frequency questionnaire [37]. This questionnaire explores the general dietary habits, cooking methods, and intake frequency of 58 foods and beverages consumed in Japan, including the daily intake of rice and miso soup, consumption frequency of non-alcoholic beverages, and the amount per drink consumed for five alcoholic beverages [36]. In this study, we asked the participants about the consumption frequency of selected foods during the previous month, without mentioning portion size, while a managerial dietician or investigator helped them complete the questionnaire. The food groups included in the BDHQ were meat, fish, vegetables, fruits, cereals, seasonings/condiments, fermented soybean paste (miso), noodle soup, confectionaries, alcoholic and non-alcoholic beverages, and dairy products. The participants were asked about the consumption frequency of each food (once a day, twice or more daily, once a week, 2–3 times a week, 4–6 times a week, less than once a week, or did not eat/drink). Using the responses for BDHQ and an ad hoc computer algorithm based on the Standard Tables of Food Composition in Japan, the daily intake of food items, mean daily intake of energy, and chosen nutrients were determined [35–37]. High sodium intake was reported to be associated with hypertension; however, potassium intake or sodium-to-potassium ratio must also be considered [57]. Hence, we calculated the sodium-to-potassium ratio from the quantities of sodium and potassium intake from the BDHQ data [40]. Although this has not been validated yet, previous studies have reported that the high potassium intake or low sodium-to-potassium ratio may have beneficial possibilities for BP [57]. Daily alcohol consumption was calculated as a part of the BDHQ [35,37]. The data used in this study were coded to preserve the anonymity of the participants.

2.7. Sample Size Calculation and Statistical Analysis

This was a prospective cohort study, and the sample size was calculated based on the data of previous studies that assessed the relationship between dietary intakes and oral functions [22,36]. Since sodium intake was found to be the factor closely related to the BP of older adults in a previous study [5], we found it appropriate to determine the sample size of this study based on the sodium intake. Based on this, the minimum number of participants required in each group was 185, and, hence, the analysis was performed with data, including those of the participants who presented to our study until December 2019 (at this time, the number of participants in each group exceeded the required number as per the sample size calculation).

The normality of the data distribution was verified using the Kolmogorov–Smirnov test ($p > 0.05$); the data were found to be non-normally distributed. To determine the differences in oral health and dietary intake among the participants with different BP groups, Kruskal-Wallis and Chi-square tests were performed, while intergroup comparisons were performed using the Mann-Whitney U-test with Bonferroni correction. The measurement items were specified according to a Kruskal-Wallis or X^2 test ($p < 0.05$, explanatory variable for basic characteristics and oral health items; $p < 0.1$, explanatory variable for dietary intake). We used the Spearman's rank correlation coefficient to examine the relationship between oral health and dietary intake. To determine the impact of hypertension-related factors, we performed binary logistic regression analyses with stepwise methods (input 0.05, remove 0.15). Hypertension, defined as participants classified as Hypertensive or History of hypertension, was considered the objective variable (Normotensive participants = 0, Hypertensive or History of hypertension participants = 1). The explanatory variables were selected from the participants' basic characteristics, oral health, and dietary intake, which were found to be significantly related to hypertension by the Kruskal-Wallis or chi-square test. All statistical analyses were performed using the SPSS Statistics 22.0 (IBM, Tokyo, Japan).

3. Results

3.1. Subject Characteristics

Table 1 provides an overview of the participants. This study included 894 participants (mean age: 74.3 ± 0.2 years old), most of whom were female (68.5%). The results also indicated that 45.3% of the participants had history of hypertension, with females accounting for 64.4% of the History of hypertension group. Overall, the aggregate prevalence of hypertension in the Hypertensive and the History of hypertension groups was 69.1%, which was relatively high. Among the three groups, statistically significant differences were found in sex, smoking habits, SBP and DBP, pulse pressure, cardiovascular disease, and stroke. In contrast, we only found significant differences between the Normotensive and the History of hypertension groups in terms of age. The Normotensive group was statistically different from the Hypertensive and the History of hypertension groups in terms of BMI, body fat mass, and percent body fat, while a significant difference between the Hypertensive and the History of hypertension groups was observed for the skeletal muscle mass. No significant differences were found in education, living arrangement, exercise habits, alcohol consumption, diabetes mellitus, dyslipidemia, and chronic kidney disease.

Table 1. Subject characteristics.

Measurement Variables	Total (*n* = 894)	Normotensive Group (*n* = 276)	Hypertensive Group (*n* = 213)	History of Hypertension Group (*n* = 405)	*p*-Value	Two-Group Comparison
Age (Years) *	74.3 ± 0.2	73.3 ± 0.3	74.3 ± 0.4	75.0 ± 0.3	0.002	b
Sex **						
Male	282 (31.5)	82 (29.7)	56 (26.3)	144 (35.6)	0.046	c
Female	612 (68.5)	194 (70.3)	157 (73.7)	261 (64.4)		
Education (years)	12.4 ± 0.1	12.4 ± 0.1	12.4 ± 0.1	12.3 ± 0.1	0.80	
Living arrangement						
Alone	98 (14.1)	33 (15.1)	24 (14.5)	41 (13.3)	0.83	
With Family	596 (85.9)	186 (84.9)	142 (85.5)	268 (86.7)		
Exercise habits	524 (58.7)	178 (64.5)	120 (56.3)	226 (55.8)	0.06	
Exercise Frequency (times)	14.1 ± 0.5	14.4 ± 0.8	12.6 ± 0.9	14.7 ± 0.7	0.29	
Exercise periods (years)	11.4 ± 0.7	10.9 ± 1.1	11.8 ± 1.5	11.7 ± 1.2	0.88	
Alcohol Consumption (g/day)	7.3 ± 0.5	6.0 ± 15.7	5.9 ± 0.9	8.8 ± 0.9	0.32	
Current or past smoking habit **	247 (27.6)	71 (25.7)	48 (22.5)	128 (31.6)	0.039	
Blood Pressure						
Systole (mmHg) *	139.3 ± 0.6	126.8 ± 0.6	152.8 ± 0.9	140.7 ± 0.9	<0.001	a b c
Diastole (mmHg) *	80.0 ± 0.4	73.5 ± 0.5	87.0 ± 0.6	80.8 ± 0.5	<0.001	a b c
Pulse Pressure (mmHg) *	59.3 ± 0.4	53.4 ± 0.5	65.8 ± 0.7	59.9 ± 0.6	<0.001	a b c
Body Composition						
BMI (kg/m^2) *	22.7 ± 0.1	21.7 ± 0.2	22.8 ± 0.2	23.3 ± 0.1	<0.001	a b
Skeletal Muscle Mass (kg) *	21.0 ± 0.1	20.6 ± 0.2	20.4 ± 0.3	21.6 ± 0.2	0.003	b c
Body Fat Mass (kg) *	15.4 ± 0.2	13.9 ± 0.3	15.8 ± 0.4	16.1 ± 0.3	<0.001	a b
Percent Body Fat *	27.8 ± 0.3	26.3 ± 0.4	28.8 ± 0.5	28.4 ± 0.4	<0.001	a b
Comorbidity Disease						
Diabetes mellitus	107 (12.0)	29 (10.5)	20 (9.4)	55 (13.6)	0.55	
Dyslipidemia	208 (23.3)	58 (21.0)	51 (23.9)	99 (24.4)	0.56	
Chronic kidney disease	27(3.0)	5 (1.8)	8 (3.8)	14 (3.5)	0.36	
Cardiovascular disease (CVD) **	61 (6.8)	17 (6.2)	7 (3.3)	37 (9.1)	0.020	
Stroke **	12 (1.3)	1 (0.4)	1 (0.5)	10 (2.5)	0.029	

Data are presented as mean ± standard error or number of participants (%). The significance level was set at $p < 0.05$. *: Significant differences were seen using the Kruskal-Wallis Test, **: Significant differences were seen using the X^2-test. BMI: body mass index; Normotensive: Participants with systolic blood pressure (SBP) < 140 mmHg and diastolic blood pressure (DBP) < 90 mmHg; Hypertensive: Participants with SBP of ≥140 mmHg and/or DBP ≥ 90 mmHg; History of hypertension: Participants who answered that they had a history of hypertension in the medical interview and/or is taking hypertension medication; Exercise habits: participants who regularly exercise; exercise frequency: the frequency of exercise every month; exercise periods: the number of years the participants have been exercising. Current or past smoking habits: Participants who smoked or had a smoking history. The Mann-Whitney U-test with Bonferroni correction was used to compare the two groups: a: statistically significant difference between the Normotensive and the Hypertensive groups; b: statistically significant difference between the Normotensive and History of hypertension groups; c: statistically significant difference between the Hypertensive and History of hypertension groups.

3.2. The Relationship between Oral Health and Hypertension

Table 2 shows the analysis of oral health parameters and their association with hypertension. The mean average of the remaining teeth among participants in the History of hypertension group was significantly lower than that of the Normotensive group, while the Normotensive group had significantly higher masticatory performance than that of the Hypertensive or the History of hypertension groups. It was found that there was a significant difference in posterior occlusal contact between the Normotensive and the History of hypertension groups. Among all the participants, 17.9% had no posterior occlusal contact, with the History of hypertension group having the highest prevalence of having no posterior occlusal contact. Other parameters such as occlusal force, oral moisture, bacterial count, and levels were not associated with BP condition. Although there was no significant difference in oral moisture, it can be noted that the History of hypertension group had the lowest oral moisture count among all the groups.

Table 2. The Relationship Between Oral Health and Hypertension.

Variables	Total (n = 894)	Normotensive Group (n = 276)	Hypertensive Group (n = 213)	History of Hypertension Group (n = 405)	p-Value	Two-Group Comparison
Remaining teeth (teeth) *	19.8 ± 0.3	21.2 ± 0.5	19.6 ± 0.6	19.1 ± 0.5	0.007	b
Maximum Occlusal force (kgf)	51.1 ± 1.3	53.5 ± 2.3	51.4 ± 2.5	49.2 ± 1.9	0.27	
Masticatory Performance (score) *	4.0 ± 0.1	4.3 ± 0.1	3.7 ± 0.2	3.9 ± 0.1	0.004	a b
Posterior occlusal contact **					0.012	b
With posterior occlusion (w/PO)	671 (75.1)	226 (81.9)	159 (74.6)	286 (70.6)		
Without posterior occlusion (w/o PO)	160 (17.9)	32 (11.6)	39 (18.3)	89 (22.0)		
Edentulous	63 (7.0)	18 (6.5)	15 (7.0)	30 (7.4)		
Oral Moisture						
Cheek	28.4 ± 0.2	28.5 ± 0.2	28.6 ± 0.7	28.2 ± 0.2	0.42	
Tongue	26.8 ± 0.1	27.1 ± 0.2	26.6 ± 0.3	26.8 ± 0.2	0.45	
Total mucus	55.2 ± 0.3	55.6 ± 0.4	55.2 ± 0.9	55.0 ± 0.4	0.43	
Bacterial count (CFU/mL)	$4.6 \times 10^7 \pm 1.7 \times 10^7$	$6.6 \times 10^7 \pm 4.3 \times 10^7$	$2.3 \times 10^7 \pm 3 \times 10^6$	$4.6 \times 10^7 \pm 2.3 \times 10^7$	0.83	
Bacterial level (min. max: 1. 7)	4.8 ± 0.03	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.04	0.97	

Data are presented as mean ± standard error or number of participants (%). The significance level was set at $p < 0.05$. * Significant differences were seen using the Kruskal-Wallis test; **: Significant differences were seen using the X^2-test. kgf: kilogram-force, CFU: colony-forming unit, min: minimum, max: maximum. Posterior occlusal contact was classified into three groups according to the availability of posterior teeth based on the Eichner index. With posterior occlusion (with/PO) group consisting of Eichner A1, A2, A3, B1, B2, and B3; Without Posterior occlusion (w/o PO) group consisting of Eichner B4, C1, and C2; the edentulous group consisting of Eichner C3 [19]. The Mann-Whitney U-test with Bonferroni correction was used to compare the two groups: a: statistically significant difference between the Normotensive and the Hypertensive groups; b: statistically significant difference between the Normotensive and History of hypertension groups; c: statistically significant difference between the Hypertensive and History of hypertension groups.

3.3. Comparison of Nutrient and Dietary Intake among Normotensive, Hypertensive, and History of Hypertension Groups

Table 3 shows the comparison between the nutrient and dietary intake among the three groups (Normotensive, Hypertensive, and History of hypertension). There were significant differences in sodium-to-potassium ratio, β-carotene, vitamin K, and meat intake. The sodium-to-potassium ratio of the Normotensive group was significantly lower than that of the other two groups, while intakes of β-carotene, Vitamin K, green and yellow vegetables, and meat were significantly lower in the History of hypertension group. Moreover, it was also found that the sodium-to-potassium ratio (1.80 ± 0.06) of males and the meat intake in the Hypertensive group was highest among the groups (Appendix A Table A3). In other words, participants in the History of hypertension group had a lower intake of green and yellow vegetables, β-carotene, and vitamin K, and higher sodium-to-potassium ratio.

3.4. Relationship among Nutrient, Food Items, and Oral Health of the Participants

Although significant differences were found among the three groups, as seen in Table 3, age was the only factor significantly correlated with β-carotene, vitamin K, green and yellow vegetables, and meat. The sodium-to-potassium ratio was found to show a statistically

significant correlation between the remaining teeth and masticatory performance; however, the correlation was weak (Table 4).

Table 3. Comparison of nutrient and dietary intake among groups.

Variables	Total (n = 894)	Normotensive Group (n = 276)	Hypertensive Group (n = 213)	History of Hypertension Group (n = 405)	p-Value	Two-Group Comparison
Nutrient Intake						
Energy (kcal/day)	2083.8 ± 21.4	2080.1 ± 38.0	2099.6 ± 46.2	2078.0 ± 31.1	0.97	
Carbohydrates (g/day)	263.6 ± 2.9	263.8 ± 5.2	265.2 ± 6.1	262.6 ± 4.3	0.99	
Protein Total (g/day)	90.8 ± 1.2	91.1 ± 2.1	92.5 ± 2.6	89.8 ± 1.7	0.93	
Animal	56.5 ± 0.9	56.0 ± 1.6	58.2 ± 2.1	56.1 ± 1.4	0.96	
Vegetable	34.3 ± 0.4	35.1 ± 0.7	34.3 ± 0.8	33.8 ± 0.5	0.47	
Fat Total (g/day)	66.1 ± 0.8	66.6 ± 1.4	67.4 ± 1.8	65.1 ± 1.2	0.65	
Animal	32.3 ± 0.5	32.3 ± 0.8	33.1 ± 1.1	31.8 ± 0.7	0.83	
Vegetable	33.9 ± 0.4	34.4 ± 0.8	34.3 ± 0.9	33.3 ± 0.6	0.59	
Sodium (mg/day)	4994.3 ± 59.5	4950.0 ± 103.6	5082.4 ± 130.4	4978.3 ± 87.1	0.95	
Potassium (mg/day)	3392.7 ± 44.0	3506.1 ± 81.0	3382.5 ± 92.9	3320.7 ± 63.0	0.34	
Sodium-to-Potassium Ratio *	1.55 ± 0.01	1.48 ± 0.02	1.56 ± 0.03	1.58 ± 0.02	0.002	a b
Calcium (mg/day)	806.0 ± 11.5	828.9 ± 21.2	797.2 ± 24.0	795.0 ± 16.7	0.39	
Magnesium (mg/day)	329.4 ± 4.1	337.4 ± 7.5	329.4 ± 8.7	323.9 ± 5.8	0.51	
Fatty Acids (g/day)						
Saturated	17.8 ± 0.2	18.0 ± 0.4	18.2 ± 0.5	17.5 ± 0.3	0.51	
Monounsaturated	23.2 ± 0.3	23.3 ± 0.5	23.8 ± 0.6	22.8 ± 0.4	0.56	
Polyunsaturated	15.7 ± 0.2	15.9 ± 0.3	15.9 ± 0.4	15.5 ± 0.3	0.79	
Dietary Fiber (g/day)	15.6 ± 0.2	16.2 ± 0.4	15.6 ± 0.4	15.2 ± 0.3	0.33	
Water-Soluble	4.0 ± 0.1	4.1 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	0.29	
Insoluble	11.1 ± 0.1	11.5 ± 0.3	11.1 ± 0.3	10.8 ± 0.2	0.29	
β-carotene (μg/day) *	5331.5 ± 103.5	5760.4 ± 206.3	5251.8 ± 200.0	5081.2 ± 145.1	0.07	b
Retinol (mg/day)	1064.4 ± 24.8	1115.0 ± 45.7	1015.2 ± 50.1	1055.3 ± 36.5	0.41	
Vit. B1 (mg/day)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.55	
Vit. B2 (mg/day)	1.7 ± 0.0	1.8 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	0.24	
Niacin (mg/day)	21.8 ± 0.3	21.9 ± 0.5	22.1 ± 0.7	21.5 ± 0.5	0.87	
Vit. B6 (mg/day)	1.7 ± 0.0	1.7 ± 0.0	1.7 ± 0.0	1.6 ± 0.0	0.73	
Vit. B12 (μg/day)	14.5 ± 0.3	14.1 ± 0.5	14.5 ± 0.6	14.8 ± 0.5	0.78	
Folate (μg/day)	470.9 ± 6.5	492.0 ± 11.9	466.4 ± 13.3	458.8 ± 9.4	0.12	
Pantothenic Acid (mg/day)	8.3 ± 0.1	8.4 ± 0.2	8.3 ± 0.2	8.2 ± 0.1	0.75	
Vit. C (mg/day)	167.3 ± 2.7	172.0 ± 4.9	167.8 ± 5.5	163.7 ± 4.0	0.51	
Vit. D (μg/day)	24.6 ± 0.6	24.3 ± 1.0	25.1 ± 1.3	24.6 ± 0.9	0.99	
Vit. K (μg/day) *	425.1 ± 7.6	451.8 ± 14.3	425.5 ± 15.8	406.7 ± 10.8	0.07	b
Food Groups (g/day)						
Cereals	371.2 ± 5.5	367.1 ± 9.5	374.9 ± 11.1	372.1 ± 8.6	0.85	
Potatoes	65.8 ± 1.9	68.2 ± 3.6	62.4 ± 3.7	66.0 ± 2.9	0.59	
Sugars and sweeteners	6.1 ± 0.1	6.0 ± 0.3	6.4 ± 0.3	5.9 ± 0.2	0.66	
Legume	89.9 ± 1.7	96.1 ± 3.5	86.3 ± 3.5	87.6 ± 2.3	0.23	
Green and yellow vegetables *	143.2 ± 2.9	154.5 ± 5.5	139.8 ± 5.9	137.3 ± 4.1	0.06	b
Other vegetables	221.6 ± 4.0	232.7 ± 7.6	220.3 ± 8.2	214.6 ± 5.7	0.21	
Fruits	162.1 ± 4.1	157.3 ± 6.7	165.0 ± 7.9	163.9 ± 6.5	0.74	
Seafood	125.5 ± 3.0	122.1 ± 5.0	128.5 ± 6.5	126.3 ± 4.4	0.90	
Meat *	80.6 ± 1.7	79.1 ± 2.9	88.9 ± 3.8	77.3 ± 2.4	0.03	c
Eggs	49.9 ± 1.1	50.2 ± 1.8	48.8 ± 2.0	50.3 ± 1.7	0.76	
Dairy Products (Milk)	184.4 ± 4.0	194.9 ± 7.7	172.7 ± 8.0	183.5 ± 5.7	0.24	
Fats and oils	11.5 ± 0.2	11.4 ± 0.3	11.9 ± 0.4	11.3 ± 0.3	0.48	
Confectionery	69.3 ± 1.9	68.6 ± 3.4	71.3 ± 4.1	68.7 ± 2.6	0.85	
Beverages	703.6 ± 11.8	737.3 ± 22.1	688.3 ± 24.4	688.7 ± 16.8	0.21	
Condiments	226.3 ± 5.0	224.5 ± 9.4	236.6 ± 10.6	222.1 ± 6.9	0.56	
Salt Intake	12.6 ± 0.2	12.5 ± 0.3	12.8 ± 0.3	12.6 ± 0.2	0.95	

Dietary assessment is the result of a brief self-administered diet history questionnaire (BDHQ). The results are expressed as the mean ± standard error. The significance level was set to $p < 0.1$. The sodium-to-potassium ratio was defined as the amount of sodium consumed divided by the amount of potassium consumed [40,57], Vit.: Vitamin. * Significant differences were observed using the Kruskal-Wallis test. Mann-Whitney's U-test with Bonferroni correction was used to compare the two groups: a: statistically significant difference between the Normotensive and the Hypertensive group; b: statistically significant difference between the Normotensive and the History of hypertension group; c: statistically significant difference between the Hypertensive group and the History of hypertension group.

Table 4. Relationship among nutrient, food items, and oral health.

Variables	Age		Remaining Teeth		Masticatory Performance	
Overall	***r***	***p*-Value**	***r***	***p*-Value**	***r***	***p*-Value**
Sodium-to-potassium ratio	0.04	0.23	−0.07 *	0.03	−0.09 *	0.01
β-carotene	0.10 **	0.003	0.04	0.28	0.03	0.46
Vitamin K	0.16 **	<0.001	0.02	0.61	0.03	0.45
Green and yellow vegetables	0.12 **	<0.001	0.03	0.32	0.03	0.38
Meat	0.09 **	0.006	0.02	0.51	0.01	0.82
Normotensive group						
Sodium-to-potassium ratio	0.08	0.16	−0.05	0.36	−0.10	0.09
β-carotene	0.11	0.08	0.01	0.92	0.03	0.63
Vitamin K	0.19 **	0.001	−0.02	0.69	−0.02	0.73
Green and yellow vegetables	0.08	0.17	0.01	0.92	0.05	0.38
Meat	0.20 **	0.001	0.01	0.87	0.00	0.95
Hypertensive group						
Sodium-to-potassium ratio	−0.03	0.62	−0.08	0.26	−0.03	0.66
β-carotene	0.15 *	0.03	0.03	0.68	0.02	0.83
Vitamin K	0.15 *	0.03	0.03	0.67	0.02	0.74
Green and yellow vegetables	0.15 *	0.03	0.05	0.43	0.02	0.74
Meat	0.03	0.61	0.06	0.37	0.07	0.34
History of hypertension group						
Sodium-to-potassium ratio	0.01	0.79	−0.05	0.31	−0.07	0.16
β-carotene	0.09	0.07	0.04	0.43	0.01	0.83
Vitamin K	0.18 **	<0.001	0.02	0.64	0.04	0.40
Green and yellow vegetables	0.15 **	0.002	0.02	0.67	0.00	0.97
Meat	0.05	0.31	0.01	0.89	0.00	0.94

r: Spearman correlation coefficient, *: $p < 0.05$, **: $p < 0.001$.

3.5. Factors Affecting Hypertension

The logistic regression analysis revealed that age, BMI, sodium-to-potassium ratio, and absence of posterior occlusal contact were significant explanatory variables affecting hypertension and/or need for BP control with medication in the participants of this study (Table 5). The risk of hypertension and/or need for BP control with medication increased with age (odds ratio (OR): 1.04; confidence interval (CI): 1.01, 1.07), it was 1.2 times higher in participants with a higher BMI (OR: 1.23; CI: 1.16, 1.30), 1.7 times in those with a higher intake of sodium-to-potassium ratio (OR: 1.65; CI: 1.12, 2.43), and 1.7 times in those without posterior occlusal contact (OR: 1.73; CI: 1.11, 2.69).

Table 5. Factors affecting hypertension.

Variables	B	Standard Error	Wald	*p*-Value	Odds Ratio	95% CI	
						Lower	Upper
Age (Years) *	0.04	0.02	6.98	0.01	1.04	1.01	1.07
BMI (kg/m^2) *	0.20	0.03	45.19	<0.001	1.23	1.16	1.30
posterior occlusal contact							
With posterior occlusion (w/PO) *	–	–	6.15	0.05	ref	–	–
Without posterior occlusion (w/o PO) *	0.55	0.23	5.86	0.02	1.73	1.11	2.69
Edentulous	−0.06	0.33	0.03	0.86	0.94	0.49	1.80
Sodium-to-potassium ratio *	0.50	0.20	6.34	0.01	1.65	1.12	2.43
intercept	−7.45	1.31	32.18	<0.001	0.001		

Binary logistic regression analyses with stepwise methods (input: 0.05; removal: 0.15). Nagelkerke $R^2 = 0.125$. Only the variables in the equation are presented. Hypertension, defined as participants belonging to the Hypertensive or the History of hypertension groups, was considered the objective variable (normotensive participants = 0, Hypertensive or History of hypertension participants = 1). The variables in Tables 1–3, which were significantly associated with hypertension, were entered as explanatory variables. B: Unstandardized coefficient; CI: 95% confidence interval for unstandardized coefficients. * Statistically significant explanatory variables, $p < 0.1$.

4. Discussion

In this study, we aimed to assess the role of oral health in nutrition and hypertension by exploring dietary intakes in a Japanese older adult population. Impaired mastication makes an individual more susceptible to developing systemic diseases that could lead to frailty and mortality when not addressed properly. Among the participants in this study, 45.3% had a history of hypertension, with the majority being females (64.4%), older, and having a higher BMI, higher alcohol consumption, exercise and smoking habits, comorbidity diseases such as diabetes mellitus, dyslipidemia, chronic kidney disease, cardiovascular disease (CVD), stroke, having a lower number of remaining teeth, loss of posterior occlusal contact, decreased occlusal force, lower oral moisture count, lower masticatory performance, lower intake of green and yellow vegetables, β-carotene, vitamin K, and high sodium-to-potassium ratio. This study showed that age was the only factor significantly associated with β-carotene, vitamin K, green and yellow vegetables, and meat, while the sodium-to-potassium ratio barely correlated with the remaining teeth and masticatory performance. Furthermore, significant explanatory variables affecting hypertension among the participants were BMI, age, sodium-to-potassium ratio, and the absence of posterior occlusal contact. According to prior findings, periodontal disease can affect the number of remaining teeth [10]. Decrease in posterior occlusal contact can lead to a decline in mastication ability [17]. Since mastication is an essential function for ingestion and digestion, a decline in mastication ability induces many functional declines, including malnutrition. If problems pertaining to nutrition are not addressed appropriately, it could lead to systemic issues, such as hypertension [17]. The results of our study postulate that decreased posterior occlusal contact possibly restricts individuals to chew foods, such as green and yellow vegetables, thus consequently decreasing dietary fiber intake, leading to low potassium and high sodium intake, which are risk factors for developing hypertension. The study findings suggest that maintaining good oral health is important to improve nutritional intake and prevent hypertension. To our knowledge, this is the first study that acknowledged oral health as another contributing factor in the development of high BP by examining the dietary intakes of the older adult in the Japanese population.

4.1. The Role of Oral Health in Nutrition and Hypertension

The findings of this study revealed that the risk of hypertension increased with age, which was consistent with previous reports [5,16]. The study also revealed that in older adults, the absence of posterior occlusal contact increased the risk of hypertension by 1.7 times. The loss of occlusal contact results in a decrease in mastication ability [17]. Thus, this study postulates that the participants in the History of hypertension group were found to have a lower masticatory performance because they had lower oral moisture count, decreased occlusal force, no posterior occlusal contact, and a lesser number of remaining teeth. Tooth loss can lead to hyperactivity of the masticatory system, leading to unhealthy diet patterns and reduced nutrient intake that can negatively influence the general health [58]. Studies have reported that tooth loss was directly related to reduced oral function [17]. Hence, the Japan Ministry of Health, Labor, and Welfare continuously promotes preserving $\geq$ 20 natural teeth until the age of 80 [58] because tooth loss can decrease posterior occlusal contact, consequently influencing the occlusal force [59]. Reduced occlusal force affects saliva secretion, which is necessary for oral functions such as mastication and swallowing. Other factors such as systemic diseases and medications can influence saliva secretion [17,60]. Hasegawa et al., found that the patients taking antihypertensive drugs have decreased saliva secretion [60]. Dry mouth is a common side effect of antihypertensive medication, which could deteriorate teeth and periodontal condition [49], eventually leading to tooth loss if not addressed properly. In this study, the participants were found to be taking calcium channel blockers (CCBs), renin–angiotensin system inhibitors (angiotensin II receptor blockers (ARBs), angiotensin-converting enzyme (ACE) inhibitors), aldosterone receptor antagonists, β-blockers, and α-blockers. Moreover, there were several participants who took two combinations of medication. It was reported that the masticatory ability

was influenced by the number of natural teeth, occlusal support, muscular weakness, and occlusal force [23].

Mastication is a necessary function for nutrition-ingestion, and an impaired mastication ability leads to many functional declines, including malnutrition [17]. Several observational studies have revealed that tooth loss, particularly in older adults, was associated with dietary intake of fruits and vegetables and a low intake of antioxidative vitamins. Furthermore, the low occlusal force has been linked to a decrease in vegetable, fruit, antioxidant, and dietary fiber intake [23]. The present study presumed that the individuals without posterior occlusal contact prefer foods that are easy to chew and avoid vegetables high in potassium, which was consistent with a study finding that Japanese older adults with <19 teeth had a significantly lower intake of vegetables than those with ≥20 [61]. This was also consistent with the findings of our study that the participants in the History of hypertension group had the lowest intake of potassium. According to the JSH 2019 guidelines, improving dietary patterns, such as increasing fruit and vegetable intakes (to 350 g/day), can reduce the risk of hypertension because potassium antagonizes the hypertensive activity of the sodium [5]. It was also pointed out that balanced ingestion of fruits and vegetables (especially green and yellow vegetables) may be necessary for improved general health [17].

4.2. Other Factors Related to Hypertension

Our findings also revealed that the participants with a higher intake of sodium-to-potassium ratio had 1.7 times higher risk of developing hypertension, which confers with the report of Park et al. [40] that sodium-to-potassium ratio and blood pressure were strongly correlated. Moreover, participants in the Hypertensive group had the highest intake of sodium (Na) and salt (NaCl), 5082.4 mg/day and 12.8 g/day, respectively, which was higher than the Japanese Society of Hypertension's recommendation of 6.0 g/day for hypertensive individuals [5]. However, because the BDHQ was designed to evaluate Japanese dietary habits and was not specific for sodium intake, we were unable to establish the relationship between salt intake, sodium intake, and hypertension in this study [57]. The current study considered that the easy-to-chew foods preferred by the older Japanese adults were processed foods with high salt content [5]. It was previously reported that the high salt intake increases BP, and excessive salt intake was one of the possible causes for the high prevalence of hypertension and stroke in Japan [5,62]. Furthermore, we also found that even if the participants in this study had a normal BMI, their BP was still higher, which was consistent with the report of the JSH 2019 that the hypertensive Japanese are often free of obesity [5]. The results of our study also suggested that the participants with a higher BMI were 1.2 times more likely to develop hypertension and/or need BP control, which was also agreeing with the JSH 2019 report that population risk for cardiovascular diseases was higher in hypertensive non-obese individuals than that in the hypertensive obese individuals [5].

Hence, the Japanese government conducted mass media-mediated public education, obligated food manufacturers to indicate salt content in food packaging, promoted nutritional labeling in school lunch/food service industries, distributed home blood pressure measurements, and required all allied health professionals to instruct patients including non-hypertensive individuals to improve their lifestyle including the balanced dietary intake, improved oral hygiene, increased physical activities (approximately 1500 step increase in the number of steps), and maintain moderate alcohol consumption. Furthermore, the Japanese government provided home-visit dental services to promote the oral health of dependent older adults and covered dental care as part of its universal health coverage [5,13]. These strategies are required to achieve the goals of reducing hypertension, CVD morbidity/mortality, and extending the healthy life expectancy of Japanese individuals.

4.3. Limitation

Several limitations must be considered when interpreting the findings of this study. First, there might have been an underestimation or overestimation of dietary intake because the BDHQ is a self-report survey designed to evaluate Japanese dietary patterns, habitual intake, cooking, and seasoning, which did not reflect the quantity of selected foods [63]. Second, the sodium-to-potassium ratio was not calculated using the urine analysis [57]. Third, we failed to find a relationship between education and living arrangements with the hypertension risk because highly educated individuals may be more knowledgeable about choosing healthy food [64], while individuals who eat alone might choose a quick and simple meal rather than a nutritionally balanced diet [65]. This might happen because we evaluated them based on their educational years rather than the educational level. Lastly, no causal relationships among dietary intake, oral health, and hypertension were established because of the observational design of the study. Hence, further studies should be conducted in the future to elucidate more on the role of oral health in nutrition.

4.4. Clinical Implications

Given the significance of oral health in the nutritional status and general health of older adults, preserving remaining teeth and improving masticatory performance is critical. This study provided evidence that decreased posterior occlusal contact increases the risk of hypertension by 1.7 times. Hence, oral rehabilitation with dentures to maintain posterior occlusal contact may be recommended for improved masticatory performance, improved dietary intake, and a lower risk of hypertension. The findings of this study will also raise awareness about lifestyle changes that can reduce the risk of hypertension, such as lower salt intake, higher fruit and vegetable intake, maintaining a healthy weight, increasing exercise habits, decreasing smoking habits, and reducing alcohol consumption.

5. Conclusions

This study suggests that the risk of hypertension increased with age, higher BMI, higher sodium-to-potassium ratio, and lesser posterior occlusal contact. Therefore, maintaining posterior occlusal contact is vital in lowering the risk of hypertension. The findings of this study will aid in the improvement of oral health, nutritional intake, and general health by reducing the development of hypertension and potentially extending the healthy life expectancy of older adults.

Author Contributions: Conceptualization, P.M., Y.H., T.O. and K.S.; methodology, Y.H., T.O. and K.S.; software, Y.H. and K.T.; validation, Y.H.; formal analysis, P.M., Y.H., M.T.S.M. and T.Y.; investigation, Y.H., K.T., H.K. (Hiroshi Kusunoki), S.T., Y.W., T.S. and H.K. (Hiromitsu Kishimoto); resources, Y.H., K.T, H.K. (Hiroshi Kusunoki), S.T., Y.W., T.S., H.K. (Hiromitsu Kishimoto) and K.S.; data curation, Y.H. and K.T.; writing—original draft preparation, P.M. and Y.H.; writing—review and editing, Y.H., T.O. and K.S.; visualization, P.M., Y.H., T.O. and K.S.; supervision, Y.H., T.O. and K.S.; project administration, Y.H. and K.S.; funding acquisition, Y.H. and K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Hyogo Dental Association, 8020 Foundation, Mitsui Sumitomo Insurance Welfare Foundation, and by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (KAKENHI) (grant no. 16KT0012 of 2016–2018 to Ken Shinmura).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Hyogo College of Medicine (approval no. Rinhi 0342). All methods were carried out in accordance with relevant guidelines and regulations.

Informed Consent Statement: Written informed consent has been obtained from all subjects involved in the study to publish this paper.

Data Availability Statement: The materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, by contacting the corresponding author without breaching patient confidentiality.

Acknowledgments: We would like to express our sincere appreciation to Masako Shiramizu at Otemae University, Colleagues in Hyogo College of Medicine Dental and Oral surgery, and all members of the FESTA (Frail Elderly in Sasayama-Tamba Area) research team (titles omitted) for their assistance in the implementation of this study (Hiroo Yoshikawa, Soji Shimomura, Koutatsu Nagai, Kyoko Sano, Masako Ito, Hatsuo Maeda, and Gaku Amano; and to everyone at the Hyogo College of Medicine Sasayama Medical Center).

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

Appendix A

Table A1. List of the Antihypertensive medications.

Number of Participants	Drug's Name	Generic Name	Therapeutic Category	Drug Classification
13	Amlodipine Tablets	Amlodipine Besilate	Long-acting calcium antagonist	calcium channel blockers
6	Micardis Tablets	Telmisartan	Bile excretion type persistent AT1 receptor blocker	angiotensin II receptor blocker
5	Blopress Tablets	Candesartan Cilexetil	Long-acting angiotensin II receptor blocker	Beta blocker
1	Bisoprolol Fumarate	Bisoprolol Fumarate	Selective β1 antagonist	Beta blocker
2	Candesartan	Candesartan Cilexetil	Long-acting angiotensin II receptor blocker	angiotensin II receptor blocker
4	Diovan OD Tablets	Valsartan	Selective AT1 receptor blocker	angiotensin II receptor blockers
1	Losartan Potassium Tablets	Losartan Potassium	Angiotensin II antagonist	angiotensin II receptor blocker
3	Azilva Tablets	Azilsartan	Long-acting AT1 receptor blocker	angiotensin II receptor blocker
4	Norvasc Tablets	Amlodipine Besilate	Long-acting calcium antagonist	calcium channel blockers
3	Unisia Combination Tablets	Candesartan CilexetilAmlodipine Besilate	Long-acting angiotensin II receptor blocker/Long-acting calcium antagonist	angiotensin II receptor blocker
3	Coniel Tablets	Benidipine Hydrochloride	Long-acting calcium antagonist	calcium channel blocker
2	Camcia Combination Tablets Ld	Candesartan Cilexetil, Amlodipine Besilate	Long-acting angiotensin II receptor blocker/Long-acting calcium antagonist	persistent angiotensin II receptor blocker/persistent calcium antagonist combination drug
2	Irbetan Tablets	Irbesartan	Long-acting angiotensin II receptor blocker	angiotensin II receptor blocker
1	Cardenalin Tablets	Doxazosin Mesilate	Alpha1 adrenoceptor antagonist	α 1 blocker
2	Olmetec OD Tablets	Olmesartan Medoxomil	High affinity AT1-receptor antagonist	angiotensin II receptor blocker
2	Nu-Lotan Tablets	Losartan Potassium	Angiotensin II antagonist	angiotensin II receptor blocker
1	Avapro Tablets	Irbesartan	Long-acting angiotensin II receptor blocker	angiotensin II receptor blocker
1	Ecard Combination Tablets	Candesartan CilexetilHydrochlorothiazide	Long-acting angiotensin II receptor blocker/Diuretic Combination agent	angiotensin II receptor blocker
1	Renivace Tablets	Enalapril Maleate	Long-acting angiotensin-converting enzyme inhibitor	angiotensin converting enzyme inhibitor
1	Teramuro Combination Tablets	Telmisartan, Amlodipine Besilate	Bile excretion type long-acting AT1 receptor blocker/Long-acting calcium antagonist	calcium channel blockers
1	Telmisartan Tablets	Telmisartan	Bile excretion type long-acting AT1 receptor blocker	angiotensin II receptor blocker
1	Adalat-L	Nifedipine	Long-acting calcium antagonist	calcium channel blocker
1	Herbesser Tablets	Diltiazem Hydrochloride	calcium antagonist	calcium channel blocker
1	Selara Tablets	Eplerenone	Selective aldosterone blocker	aldosterone receptor antagonists
1	Spironolactone Tablets	Spironolactone	Anti-aldosterone diuresis	aldosterone receptor antagonists

AT1: Angiotensin II type 1. Hypertension medications were collected based on the medical interview gathered from the participants' information.

Table A2. List of two combinations of antihypertensive drugs.

Number of Participants	Drug's Name
1	Amlodipine Tablets and Losartan Potassium Tablets
2	Amlodipine Tablets and Micardis Tablets
1	Amlodipine Tablets and Candesartan
1	Diovan OD Tablets and Norvasc Tablets
1	Azilva Tablets and Norvasc Tablets
1	Norvasc Tablets and Cardenalin Tablets
1	Coniel Tablets and Spironolactone Tablets

OD: Orally Disintegrating. Hypertension medications were collected based on the medical interview gathered from the participants' information.

Table A3. Comparison of nutrient and dietary intake among groups by Sex.

Measurement Variables	Normotensive Group (*n* = 276)	Hypertensive Group (*n* = 213)	History of Hypertension (*n* = 405)	*p*-Value	Two-Group Comparison
Male					
Age (Years)	74.7 ± 0.7	75.0 ± 0.8	74.9 ± 0.5	0.9	b
Blood Pressure					
Systole (mmHg) *	125.8 ± 1.1	149.7 ± 1.5	138.7 ± 1.4	<0.001	a b c
Diastole (mmHg) *	73.1 ± 0.8	86.0 ± 1.1	79.6 ± 1.0	<0.001	a b c
Pulse Pressure (mmHg) *	52.7 ± 0.9	63.7 ± 1.3	59.1 ± 1.0	<0.001	a b c
Body Composition					
BMI (kg/m^2) *	22.1 ± 0.3	23.4 ± 0.3	23.7 ± 0.2	<0.001	a b
Skeletal Muscle Mass (kg) *	25.5 ± 0.3	25.3 ± 0.5	26.5 ± 0.3	0.02	c
Body Fat Mass (kg) *	12.9 ± 0.6	15.4 ± 0.7	15.2 ± 0.5	<0.001	a b
Percent Body Fat *	21.2 ± 0.7	24.5 ± 0.8	23.4 ± 0.5	0.002	a b
Comorbidity Disease					
Cardiovascular disease	11 (13.4)	1 (1.8)	18 (12.5)	0.06	
Stroke	1 (1.2)	1 (1.8)	7 (4.9)	0.26	
Current or past smoking habits	71 (25.7)	48 (22.5)	128 (31.6)	0.17	
Nutrient and Food Groups					
Sodium-to-potassium ratio *	1.6 ± 0.1	1.8 ± 0.1	1.7 ± 0.04	0.01	a
β-carotene (μg/day)	5058.5 ± 308.6	4543.2 ± 380.5	4764.5 ± 241.3	0.47	
Vit. K (μg/day)	443.1 ± 26.2	397.0 ± 30.2	427.0 ± 19.9	0.52	
Green and yellow vegetables (g/day)	137.7 ± 8.7	124.0 ± 11.4	131.9 ± 6.9	0.53	
Meat (g/day)	77.0 ± 4.9	83.6 ± 6.6	75.3 ± 4.2	0.53	
Female					
Age (Years) *	72.8 ± 0.4	74.0 ± 0.4	75.1 ± 0.4	<0.001	b
Blood Pressure					
Systole (mmHg) *	127.2 ± 0.7	153.9 ± 1.0	141.8 ± 1.2	<0.001	a b c
Diastole (mmHg) *	73.6 ± 0.5	87.3 ± 0.8	81.5 ± 0.6	<0.001	a b c
Pulse Pressure (mmHg) *	53.7 ± 0.5	66.6 ± 0.9	60.3 ± 0.7	<0.001	a b c
Body Composition					
BMI (kg/m^2) *	21.5 ± 0.2	22.6 ± 0.2	23.1 ± 0.2	<0.001	a b
Skeletal Muscle Mass (kg)	18.5 ± 0.1	18.7 ± 0.1	18.8 ± 0.1	0.32	
Body Fat Mass (kg) *	14.4 ± 0.4	16.0 ± 0.4	16.7 ± 0.3	<0.001	a b
Percent Body Fat *	28.4 ± 0.5	30.3 ± 0.6	31.2 ± 0.4	<0.001	a b
Comorbidity Disease					
Cardiovascular disease	6 (3.1)	6 (3.8)	19 (7.3)	0.09	
Stroke	0	0	3 (1.1)	0.13	
Current or past smoking habits	71 (25.7)	48 (22.5)	128 (31.6)	0.22	

Table A3. *Cont.*

Measurement Variables	Normotensive Group (n = 276)	Hypertensive Group (n = 213)	History of Hypertension (n = 405)	p-Value	Two-Group Comparison
Nutrient and Food Groups					
Sodium to potassium ratio	1.4 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	0.08	
β-carotene (μg/day)	6057.1 ± 260.4	5504.6 ± 232.4	5255.9 ± 181.0	0.13	
Vit. K (μg/day)	455.5 ± 17.2	435.7 ± 18.5	395.5 ± 12.6	0.05	b
Green and yellow vegetables (g/day)	161.6 ± 6.9	145.4 ± 6.8	140.3 ± 5.2	0.09	
Meat (g/day)	80.0 ± 3.5	90.7 ± 4.6	78.3 ± 3.0	0.08	

Data are presented as mean ± standard error or number of participants (%). The significance level was set at $p < 0.1$. *: Significant differences were seen using the Kruskal-Wallis Test. BMI: body mass index; Vit.: Vitamin; the Normotensive: Participants with systolic blood pressure (SBP) < 140 mmHg and diastolic blood pressure (DBP) < 90 mmHg; Hypertensive: Participants with SBP of ≥140 mmHg and/or DBP ≥ 90 mmHg; History of hypertension: Participants who answered that they had a history of hypertension in the medical interview and/or is taking hypertension medication; Current or past smoking habits: Participants who smoked or had a history of smoking. The sodium-to-potassium ratio was defined as the amount of sodium consumed divided by the amount of potassium consumed [40,57]. The Mann-Whitney U-test with Bonferroni correction was used to compare the two groups: a: statistically significant difference between the Normotensive group and the Hypertensive group; b: Statistically significant difference between the Normotensive and the History of hypertension groups; c: Statistically significant difference between the Hypertensive and the History of hypertension groups.

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Combined Citrulline and Glutathione Supplementation Improves Endothelial Function and Blood Pressure Reactivity in Postmenopausal Women

Arturo Figueroa [1,*], Arun Maharaj [1,2], Yejin Kang [1], Katherine N. Dillon [1], Mauricio A. Martinez [1], Masahiko Morita [3], Dai Nogimura [3] and Stephen M. Fischer [1]

[1] Department of Kinesiology and Sport Management, Texas Tech University, Lubbock, TX 79409, USA
[2] Department of Epidemiology and Cancer Control, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
[3] Research & Development Division, KIRIN Central Research Institute, Kirin Holdings Co., Ltd., 2-26-1, Muraoka-Higashi, Fujisawa 251-8555, Kanagawa, Japan
* Correspondence: arturo.figueroa@ttu.edu; Tel.: +1-(806)-834-5587; Fax: +1-(806)-742-1688

Abstract: Postmenopausal women (PMW) may experience endothelial dysfunction associated with arginine (ARG) deficiency relative to asymmetric dimethylarginine (ADMA) caused by oxidative stress. Endothelial dysfunction contributes to increased blood pressure (BP) responsiveness to sympathoexcitation induced by the cold pressor test (CPT). We investigated the effects of citrulline alone (CIT) and combined with the antioxidant glutathione (CIT+GSH) on vascular function. Forty-four healthy PMW were randomized to CIT (6 g), CIT+GSH (2 g + 200 mg: Setria®) or placebo (PL) for 4 weeks. Brachial artery flow-mediated dilation (FMD), aortic stiffness (pulse wave velocity, PWV), brachial and aortic BP reactivity to CPT, and serum fasting blood glucose (FBG), ARG, and ARG/ADMA ratio were measured. Baseline FBG was higher in CIT+GSH vs. PL. FMD increased after CIT+GSH vs. PL ($p < 0.05$). CIT and CIT+GSH increased ARG/ADMA ($p < 0.05$), but did not affect aortic PWV. CIT+GSH attenuated the brachial and aortic systolic BP and mean arterial pressure (MAP) responses to CPT vs. PL and CIT ($p < 0.05$). The improvements in FMD were related to baseline FMD ($r = -0.39$, $p < 0.05$) and aortic MAP response to CPT ($r = -0.33$, $p < 0.05$). This study showed that CIT+GSH improved FMD and attenuated systolic BP and MAP reactivity in PMW. Although CIT increased ARG/ADMA, it did not improve FMD in healthy PMW.

Keywords: arginine/ADMA ratio; blood pressure responsiveness; citrulline; endothelial function; glutathione

Citation: Figueroa, A.; Maharaj, A.; Kang, Y.; Dillon, K.N.; Martinez, M.A.; Morita, M.; Nogimura, D.; Fischer, S.M. Combined Citrulline and Glutathione Supplementation Improves Endothelial Function and Blood Pressure Reactivity in Postmenopausal Women. *Nutrients* **2023**, *15*, 1557. https://doi.org/10.3390/nu15071557

Academic Editors: Shane Phillips and Abeer M. Mahmoud

Received: 16 February 2023
Revised: 7 March 2023
Accepted: 21 March 2023
Published: 23 March 2023

1. Introduction

The endothelium has a major role in the regulation of vascular function and structure by releasing vasodilatory molecules [1]. Nitric oxide (NO), the main vasodilator, is produced from the amino acid L-arginine (ARG) by endothelial NO synthase (eNOS) [2] in response to increased shear stress induced by blood flow. NO is the primary endothelial factor for relaxing vascular smooth muscle cells and preventing pathological structure remodeling that leads to arterial stiffness [1,3]. Reduced NO availability, a main cause of endothelial dysfunction [1,3], is associated with ARG deficiency driven by increased levels of endogenous inhibitors of eNOS [4] and ARG catabolism to ornithine (ORN) by the enzyme arginase [5,6]. Oxidative stress is a main mechanism of endothelial dysfunction via increasing asymmetric dimethylarginine (ADMA, an eNOS inhibitor) levels [7–9] and arginase activity [10]. Since ARG and ADMA compete for binding to eNOS, a low ARG/ADMA ratio is a biomarker of reduced ARG availability and NO production [7].

Impaired endothelial function, assessed as low brachial artery flow-mediated dilation (FMD), begins before and progresses after menopause in healthy women [4,11]. Endothelial

dysfunction precedes the development of arterial stiffness [12] and hypertension in healthy postmenopausal women [13]. Another mechanism for hypertension in postmenopausal women is elevated sympathetic activity [14]. Systolic hypertension and consequent heart failure with preserved ejection fraction are highly prevalent in older women due to increased aortic stiffness [15] and sympathetic-mediated vasoconstriction [16,17]. Evidence indicates that augmented systolic blood pressure (BP) reactivity to a sympathetic stimulus (cold pressor test, CPT) predicts future hypertension [18] and heart failure [19]. Exaggerated BP reactivity to sympathetic stimuli seen in postmenopausal women [16], may be due to the inability of the endothelium to counteract sympathetic-mediated vasoconstriction [17,20].

Dietary strategies to increase circulating ARG may improve vascular function. The effectiveness of short-term oral ARG supplementation for improving FMD was observed in healthy older adults [21]. However, oral ARG becomes ineffective for vascular benefits due to stimulation of arginase activity [22]. Contrary to ARG, citrulline (CIT) is not catabolized by arginase and inhibits arginase activity [23], and thus, is more efficient than oral ARG in increasing circulating ARG levels [24]. Short-term CIT supplementation has been shown to improve plasma ARG, ARG/ADMA, arterial stiffness, and resting BP in middle-aged and older adults [23,25–29]. Evidence supports the benefit of CIT supplementation on the attenuation of BP reactivity to CPT in healthy adults [30–32]. A placebo-controlled study examined the effect of CIT supplementation on FMD but failed to detect improvements in healthy adults [24]. Since oxidative stress is implicated in endothelial dysfunction, the addition of the antioxidant glutathione (GSH) may synergize the vascular benefits of CIT. Recent evidence supports the combined use of CIT and GSH (CIT+GSH) to improve eNOS activity in a mouse ischemia model [33] and the long-lasting increase of plasma NO levels in young men [34]. Although combined CIT+GSH supplementation increased post-exercise plasma NO metabolites (NOx), an indicator of improved endothelial function [34], vascular function measures were not evaluated. Therefore, it is unknown whether CIT+GSH supplementation can improve vascular function and BP reactivity in postmenopausal women. Given that CIT supplementation increased circulating ARG levels and the ARG/ADMA ratio, an indicator of increased NO availability [27], improvements in brachial FMD are expected in postmenopausal women.

The purpose of this study was to investigate the effects of supplementing with CIT alone and CIT+GSH on vascular function, assessed by brachial FMD, arterial stiffness (pulse wave velocity, PWV), and BP reactivity to CPT in postmenopausal women. Moreover, we assessed fasting blood glucose (FBG), serum levels of ARG and its metabolites, and markers of oxidative stress. Our hypothesis was that CIT alone and CIT+GSH supplementations would increase brachial FMD and attenuate BP responses to sympathetic activation induced by CPT compared to a placebo in postmenopausal women.

2. Materials and Methods

2.1. Participants

Participants were healthy postmenopausal women aged 51–74 years. All participants had absence of menstruation for at least 1 year and were sedentary (<120 min/week of exercise). Potential participants were excluded if they had diagnosed cardiovascular diseases (CVDs), type 2 diabetes, a body mass index > 34.9 kg/m^2, brachial BP > 150/90 mmHg, consumed > 7 alcoholic drinks per week, and used tobacco, hormone replacement therapy, or dietary supplements with vasodilatory and/or antioxidant effects. The rationale for excluding women with a BP > 150/90 mmHg and > 7 alcoholic drinks was the increased CVD risk associated with these levels [35,36]. The study was registered in ClinicalTrials.gov (Identifier: NCT04672447) and the protocol was approved by the Texas Tech University Institutional Review Board.

2.2. Experimental Protocol

This was a double-blind, placebo-controlled, parallel design study. The randomization was performed by a researcher not involved in laboratory measurements using a block

scheme stratified by age and systolic BP (SBP) with a computer program [37]. Forty-four participants were randomly assigned to a daily supplementation with a placebo (crystalline cellulose) (n = 17), CIT (6 g) (n = 13) or CIT+GSH (2 g + 200 mg: Setria®) (n = 14) (Figure 1). Participants consumed 9 capsules in the morning and evening for 4 weeks (Kyowa Hakko Bio Co., Ltd., Tokyo, Japan). Each capsule containing CIT, CIT+GSH, and placebo was indistinguishable by size, shape, and taste. Both research staff and participants were blinded to the group allocation until the completion of data analysis. Participants were instructed to avoid ARG or CIT rich containing foods (e.g., watermelon, salmon, nuts [walnuts, almonds], turkey breast) or supplements with NO precursors or antioxidants during the study period. Participants were recommended to maintain their usual diet and physical activity throughout the study. The last capsules were consumed 10–12 h before their laboratory visit at the end of the study. Participants returned their bottles during the last visit and adherence was assessed by capsule count.

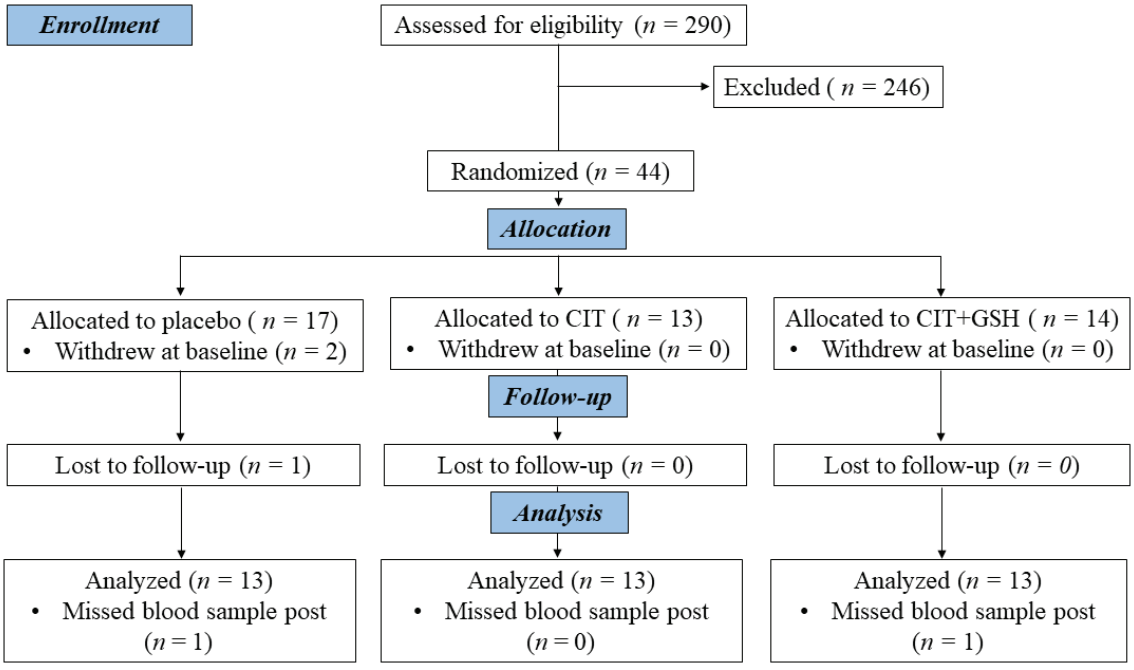

Figure 1. CONSORT flow chart of participants through the study. CIT, citrulline, CIT+GSH, and CIT+glutathione.

The primary endpoint was the change in endothelial function, as assessed by brachial FMD. The secondary endpoints were reductions in PWV at rest and BP responses to CPT. Tertiary endpoints were changes in serum glucose, insulin, ARG, CIT, ORN, ADMA, arginase I, and oxidative markers.

Participants completed a health-history questionnaire and were familiarized with the protocol. Height and weight were measured using a stadiometer and beam scale (free-standing portable height rod and weigh beam, Detecto, Webb City, MO, USA), and waist circumference was measured using a non-elastic tape. Body mass index was calculated as weight (kg) divided by height squared (m^2) and body composition was assessed using a total body dual-energy X-ray absorptiometry scanner (GE Lunar Prodigy; GE Healthcare, Madison, WI, USA).

2.3. Measurements

All measurements were performed in the morning after an overnight fast and 24 h abstinence from caffeine, alcohol, prescription medications, and physical activity. All vascular measurements were conducted in the supine position after at least 20 min of rest in a quiet, dimly lit, and thermoneutral environment. Following 4 weeks of intervention, all measurements were repeated in the same order and conducted at baseline.

2.3.1. Flow-Mediated Dilation

Endothelial function was assessed by brachial FMD. The right brachial artery was scanned ~2–3 cm proximal to the antecubital fossa using a 12-MHz linear array Doppler ultrasound probe (LogiQ S7 expert, GE Medical Systems, Milwaukee, WI, USA) at an insonation angle < 60°. Following a 2 min baseline diameter and mean blood velocity recording, the occlusion cuff on the proximal forearm was rapidly inflated to 250 mmHg using an automated pump (E20, Hokanson, Bellevue, WA, USA). Following a 5 min occlusion period, the cuff was rapidly deflated while the diameter and mean blood velocity were continuously recorded for 3 min using open-source software (OBS Studio). Baseline and peak diameters were measured using an automated edge-detection software (Quipu Cardiovascular Suite, Pisa, Italy). Baseline diameter was measured as the average vessel diameter during the 2 min baseline, and peak diameter as the largest diameter detected post-occlusion. FMD is expressed as a percentage change from the baseline diameter: FMD% = ([peak diameter − baseline diameter]/baseline diameter) × 100.

2.3.2. Pulse Wave Velocity

Carotid-femoral PWV (cfPWV, aortic), carotid-radial PWV (crPWV, brachial), carotid-distal PWV (cdPWV, systemic), and femoral-ankle (dorsalis pedis artery) PWV (faPWV, leg) were determined by applanation tonometry (Complior Analyse, Alam Medical, Vincennes, France; for faPWV: SphygmoCor CPV, AtCor Medical, Sydney, Australia). Distances between arterial sites in each segment were measured with a segmometer (Mitutoyo, Aurora, IL, USA). PWV was calculated as the distance of the arterial segment divided by the transit time of the pulse. cfPWV was multiplied by 0.80 to account for the distance between the carotid artery site and the suprasternal notch [38]. A minimum of two PWV measurements were obtained and the average was used for analysis.

2.3.3. Brachial and Aortic Blood Pressures at Rest and During the CPT

Brachial SBP and diastolic BP (DBP) were measured using an automated oscillometric device (HEM-705CP; Omron Healthcare, Vernon Hill, IL, USA). Radial artery pressure waves recorded using applanation tonometry were calibrated with brachial mean arterial pressure (MAP) and DBP. Aortic hemodynamics were obtained via a validated transfer function (SphygmoCor CPV; AtCor Medical, Sydney, Australia). A minimum of two high-quality readings (operator index ≥ 80%) were obtained at rest and during the CPT and the average was used for analysis. The participants introduced their right hand up to the wrist in cold water at 1–4 °C for 2 min. The increase in BP from rest to the second minute of the CPT (Δ) was used for the analysis, since greater sympathetic activity occurs at this time point [16].

2.3.4. Serum Biomarkers

Fasted blood samples were collected from the antecubital vein at baseline and at 4 weeks. Serum samples were collected using silicone-coated tubes and stored at −80 °C until subsequent analysis of the biomarkers. Glucose (EIAGLUC; Invitrogen, Carlsbad, CA, USA) was measured using the glucose oxidase method. Insulin was measured using an ELISA kit (80-INSHUE01.1, ALPCO Diagnostics, Salem, NH, USA). The homeostatic model assessment for insulin resistance was calculated as [fasting glucose (mg/dL) × fasting insulin (μIU/mL)]/405. Glutathione peroxidase (ab102530; Cambridge, UK), malondialdehyde (Abcam 233571; Cambridge, UK), and superoxide dismutase (ab119520; Cambridge,

UK) were analyzed using the colorimetric method. Arginase-1 (BMS2216; Invitrogen, Carlsbad, CA, USA) and oxidized low-density lipoprotein (CSB-E07931h; Cusabio Biotech, Houston, TX, USA) were measured using ELISA kits. Serum ARG, CIT, ORN, and ADMA were analyzed using an AbsoluteIDQ p400 HR kit (Biocrates, Innsbruck, Austria), which is a combined flow injection and high-performance liquid chromatography–tandem mass spectrometry assay. The tandem mass spectrometry platform consisted of a Thermo Scientific Vanquish HPLC coupled to the Thermo Scientific Q-Exactive HF Quadrupole-Obitrap mass spectrometer.

2.4. Statistical Analysis

Sample size was estimated based on data that showed increased FMD after ARG supplementation in older adults [21]. It was estimated that 12 participants would be appropriate to detect a difference with ≥80% power at the $\alpha = 0.05$ level. The Shapiro–Wilk test was used to verify the normal distribution of the data. A one-way analysis of variance was used to detect between-group differences. A two-way analysis of variance with repeated measures and Bonferroni adjustments were performed to detect time (before and after) and between-groups (placebo, CIT and CIT+GSH) differences in measures at rest and BP responses (Δ) to CPT. If significant time-by-group interactions were detected, Tukey and paired-t tests were used as post-hoc tests. Pearson's correlation coefficient was used to evaluate relationships between changes in FMD% from 0 to 4 weeks and baseline FMD, and with aortic MAP responses to CPT from 0 to 4 weeks. Independent sample t-tests were used to evaluate low (0.20), moderate (0.50), and large (≥0.80) effect sizes using Cohen's d on the changes in FMD between CIT vs. placebo, CIT+GSH vs. CIT, and CIT+GSH vs. placebo. Statistical analyses were performed using SPSS Ver26 (IBM SPSS, Armonk, NY, USA). Results are reported as the mean ± standard deviation (SD) in tables and the mean ± standard error (SE) in figures. Significance was set a priori at $p < 0.05$.

3. Results

3.1. Participant Characteristics

Participants were recruited from June 2020 to December 2021. Thirty-nine participants randomized to the placebo ($n = 13$), CIT ($n = 13$), and CIT+GSH ($n = 13$) completed the study (Figure 1). Compliance to the supplements were 94.9 ± 1.3%, 95.7 ± 1.1%, and 95.5 ± 1.2% for placebo, CIT, and CIT+GSH groups, respectively. No adverse effects of the supplementations were reported by participants during the study.

Participant characteristics at baseline did not differ among the groups, except for FBG (Table 1). FBG was higher in CIT+GSH compared to the placebo but not to CIT ($p = 0.29$).

Table 1. Participant characteristics and treatments.

Measures and Medication	Placebo ($n = 13$)	CIT ($n = 13$)	CIT+GSH ($n = 13$)	p
Age (years)	60 ± 5	58 ± 4	58 ± 6	0.60
Height (m)	1.58 ± 0.06	1.59 ± 0.08	1.59 ± 0.10	0.92
Body weight (kg)	73.3 ± 9.3	72.1 ± 11.2	75.5 ± 15.7	0.94
BMI (kg/m^2)	29.3 ± 3.4	29.0 ± 4.8	29.5 ± 4.2	0.91
WC (cm)	93.3 ± 8.5	93.1 ± 12.6	91.5 ± 11.0	0.65
Total fat mass (%)	45 ± 5	42 ± 7	46 ± 5	0.18
Total lean mass (%)	54 ± 5	55 ± 5	56 ± 6	0.18
FBG (mg/dL)	87 ± 7	90 ± 6	94 ± 7 †	0.04
Medication (*n*)				
ARB/ACE inhibitors	2	1	3	
Diuretics	1	0	2	
Ca^{2+} channel blockers	1	0	0	

Values are the mean ± SD or number (n) of participants. Abbreviations: BMI, body mass index; WC, waist circumference; FBG, fasting blood glucose; CIT, citrulline; GSH, glutathione; ARB, angiotensin receptor blocker; ACE, angiotensin converting enzyme; † vs. placebo.

3.2. Flow-Mediated Dilation and Arterial Stiffness

Baseline FMD and PWV segments did not differ among the groups. There was a time-by-group interaction for FMD. CIT+GSH increased the FMD by 2.9% ($p = 0.045$) compared with placebo, but not with CIT ($p = 0.18$) (Figure 2). A low effect size was seen between CIT and placebo ($d = 0.32$) and, although insignificant ($p = 0.10$), there was a moderate effect size between CIT+GSH and CIT ($d = 0.67$). To compliment the significant increase in FMD, there was a high effect size between CIT+GSH and placebo ($d = 0.91$, $p = 0.03$). The change in FMD from 0–4 weeks was correlated with the baseline FMD (Figure 3). The Pearson correlation coefficient for placebo, CIT, and CIT+GSH were -0.26 ($p = 0.39$), 0.16 ($p = 0.60$), and -0.68 ($p = 0.01$), respectively. There were no significant time-by-group interactions for cfPWV, crPWV, cdPWV, or faPWV. However, CIT+GSH supplementation decreased crPWV (arm PWV) by 0.66 ± 0.93 m/s ($p = 0.05$, Table 2).

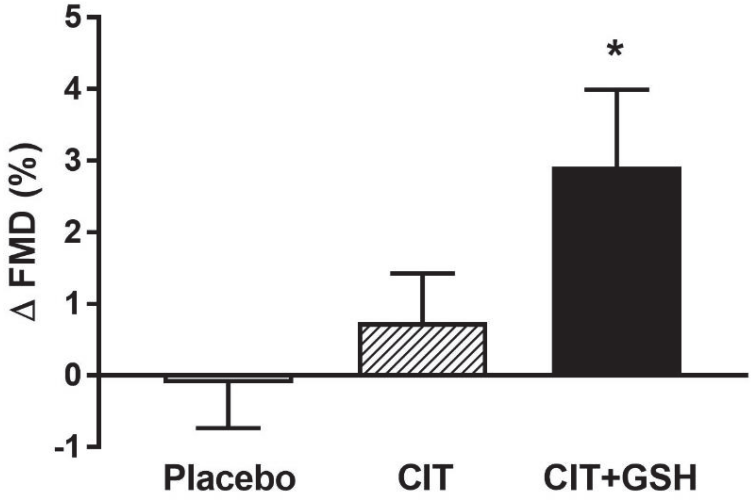

Figure 2. Changes (Δ) in brachial artery flow-mediated dilation (ΔFMD%) from 0 to 4 weeks. Abbreviations: CIT, citrulline; CIT+GSH, CIT+glutathione. * $p < 0.05$ vs. placebo.

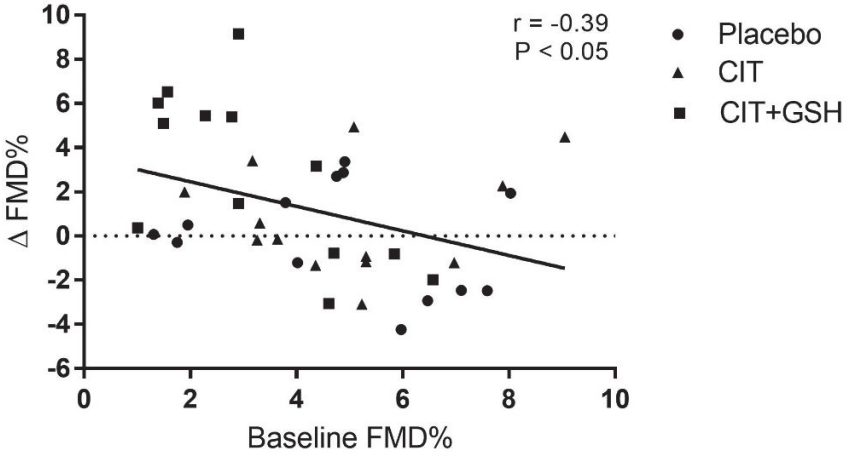

Figure 3. Relationship between changes (Δ) in the brachial artery flow-mediated dilation (ΔFMD%) from 0 to 4 weeks and baseline (0 week) FMD%. Abbreviations: CIT, citrulline; CIT+GSH, CIT+glutathione.

Table 2. Brachial artery endothelial function and arterial stiffness.

Measure	Placebo		CIT		CIT+GSH		
	Before	After	Before	After	Before	After	*p*
FMD (%)	4.8 ± 2.2	4.8 ± 2.9	5.0 ± 2.0	5.7 ± 3.5	3.1 ± 1.8	6.0 ± 3.0 *‡	0.04
cfPWV (m/s)	7.6 ± 1.4	7.6 ± 1.1	6.9 ± 0.8	7.3 ± 0.8	7.3 ± 0.7	7.3 ± 1.3	0.28
crPWV (m/s)	8.3 ± 1.7	8.3 ± 1.2	8.5 ± 1.2	8.5 ± 1.8	8.5 ± 0.9	7.9 ± 0.9 *	0.51
cdPWV (m/s)	8.9 ± 1.5	8.2 ± 1.1	8.8 ± 0.8	8.4 ± 0.9	8.2 ± 0.9	8.2 ± 1.1	0.23
faPWV (m/s)	8.5 ± 0.3	8.7 ± 0.3	9.0 ± 0.3	9.3 ± 0.3	9.0 ± 0.3	8.8 ± 0.3	0.69

Values are the mean ± SD. Abbreviations: cfPWV, carotid-femoral PWV; crPWV, carotid-radial PWV; cdPWV, carotid-distal PWV; CIT, citrulline; CIT+GSH, CIT+glutathione; faPWV, femoral-ankle PWV. *p*-values are the time-by-group interaction from two-way repeated measures ANOVA. * $p < 0.05$ vs. before; ‡ $p < 0.05$ vs. placebo.

3.3. Blood Pressure at Rest and During the Cold Pressor Test

Table 3 shows the BP at rest and during the CPT before and after the supplementations. There were no significant between-group differences in the resting BP at baseline and no time-by-group interactions. There were significant time-by-group interactions for changes (Δ) in brachial SBP ($p = 0.02$), brachial MAP ($p = 0.04$), aortic SBP ($p = 0.03$), and aortic MAP ($p = 0.04$) responses to the CPT. CIT+GSH supplementation reduced ΔSBP compared to the placebo and CIT ($p < 0.05$ for both), and reduced ΔMAP compared to the placebo ($p < 0.05$) but not to CIT (Figure 4). No significant time-by-group interactions were observed for ΔDBP, Δ augmentation index normalized to a heart rate of 75, and Δ reflection time (Tr). Attenuation of aortic ΔMAP was significantly related to the improvement in FMD% ($r = -0.33$, $p < 0.05$).

Table 3. Brachial and aortic blood pressures at rest and during CPT.

Measure	Condition	Placebo		CIT		CIT+GSH		
		Before	After	Before	After	Before	After	*p*
Brachial SBP (mmHg)	Rest	113 ± 13	111 ± 12	113 ± 13	114 ± 11	110 ± 12	115 ± 12	0.27
	CPT	135 ± 15	136 ± 12	127 ± 13	132 ± 14	141 ± 18	135 ± 16	
Brachial DBP (mmHg)	Rest	75 ± 8	75 ± 8	71 ± 9	71 ± 9	71 ± 11	73 ± 8	0.69
	CPT	91 ± 11	93 ± 8	81 ± 9	83 ± 8	88 ± 14	89 ± 10	
Brachial MAP (mmHg)	Rest	88 ± 9	87 ± 9	85 ± 10	85 ± 7	84 ± 11	87 ± 7	0.40
	CPT	105 ± 11	107 ± 9	96 ± 9	99 ± 9	106 ± 14	103 ± 11	
Aortic SBP (mmHg)	Rest	107 ± 13	105 ± 11	108 ± 13	109 ± 10	104 ± 11	107 ± 9	0.35
	CPT	129 ± 15	129 ± 12	121 ± 14	125 ± 13	133 ± 18	128 ± 16	
Aortic DBP (mmHg)	Rest	76 ± 8	76 ± 8	72 ± 9	72 ± 9	72 ± 11	74 ± 8	0.66
	CPT	92 ± 11	94 ± 8	82 ± 9	83 ± 8	90 ± 14	90 ± 10	
Aortic MAP (mmHg)	Rest	87 ± 9	86 ± 9	84 ± 10	84 ± 8	83 ± 11	85 ± 7	0.70
	CPT	104 ± 11	106 ± 9	95 ± 10	97 ± 9	104 ± 14	102 ± 11	
AIx75 (%)	Rest	27 ± 6	28 ± 7	30 ± 8	29 ± 8	27 ± 5	27 ± 6	0.88
	CPT	33 ± 6	31 ± 7	32 ± 8	32 ± 6	33 ± 8	30 ± 7	
Tr (ms)	Rest	137 ± 6	138 ± 7	135 ± 8	135 ± 8	138 ± 9	136 ± 7	0.29
	CPT	135 ± 8	139 ± 11	135 ± 8	136 ± 10	138 ± 8	139 ± 8	

Values are the mean ± SD. Abbreviations: AIx, augmentation index normalized to the heart rate of 75; CIT, citrulline; CIT+GSH, CIT+glutathione; DBP, diastolic blood pressure; MAP, mean arterial pressure; SBP, systolic blood pressure; Tr, reflection time. *p*-values of ANOVA time-by-group interaction.

3.4. Serum Biomarkers

Table 4 shows serum biomarkers before and after the interventions. Significant time-by-group interactions were observed for ARG ($p = 0.005$), ORN ($p = 0.04$), and ARG/ADMA ratio ($p = 0.007$). ARG and ARG/ADMA ratios were significantly increased by CIT supplementation compared with placebo ($p = 0.01$ for both) and CIT+GSH ($p = 0.008$ and $p = 0.04$, respectively) (Figure 5A,D). The ARG/ADMA ratio significantly increased after CIT+GSH ($p = 0.04$). ORN was increased after CIT compared to CIT+GSH ($p = 0.05$) but not to the placebo ($p = 0.12$). Arginase I levels decreased after CIT ($p = 0.01$) and tended ($p = 0.07$) to

decrease after CIT+GSH, but there was no significant time-by-group interaction. Glucose, insulin, homeostatic model assessment for insulin resistance, glutathione peroxidase, superoxide dismutase, oxidized LDL, and malondialdehyde did not significantly change after 4 weeks in any group.

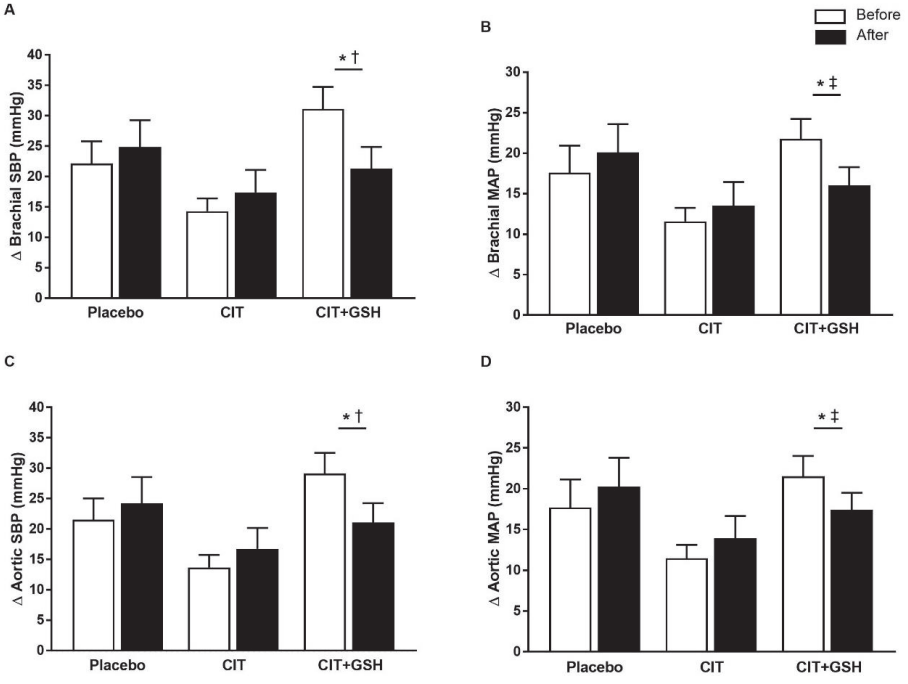

Figure 4. Changes (Δ) in (**A**) the brachial systolic blood pressure SBP (SBP), (**B**) brachial mean arterial pressure (MAP), (**C**) aortic SBP, and (**D**) aortic MAP to the cold pressor test before and after supplementations. Abbreviations: CIT, citrulline; CIT+GSH, CIT+glutathione. * $p < 0.05$ before vs. after, † $p < 0.05$ vs. placebo and CIT, ‡ $p < 0.05$ vs. placebo.

Table 4. Serum markers of arginine metabolism, glucose control, and oxidative stress.

Measure	Placebo		CIT		CIT+GSH		
	Before	After	Before	After	Before	After	*p*
Arginine (μM/L)	127 ± 21	135 ± 32	121 ± 21	168 ± 36 *†	117 ± 25	128 ± 19	0.005
Citrulline (μM/L)	41 ± 10	41 ± 13	40 ± 8	49 ± 13 *	38 ± 10	39 ± 7	0.09
Ornithine (μM/L)	106 ± 28	120 ± 34	110 ± 20	149 ± 38 ‡#	104 ± 25	114 ± 24	0.043
ADMA (mM/L)	0.54 ± 0.05	0.55 ± 0.05	0.54 ± 0.06	0.56 ± 0.06 *	0.55 ± 0.07	0.54 ± 0.08	0.27
Arginine/ADMA	240 ± 50	248 ± 61	228 ± 50	299 ± 48 *†	215 ± 47	237 ± 37 *	0.007
Arginase-I (ng/mL)	87 ± 2	87 ± 2	88 ± 3	87 ± 3 ‡	90 ± 3	89 ± 3	0.32
Glucose (mg/dL)	87 ± 7	87 ± 7	90 ± 6	91 ± 10	94 ± 7	94 ± 8	0.89
Insulin (mIU/mL)	15 ± 4	14 ± 4	15 ± 6	15 ± 5	12 ± 4	12 ± 5	0.75
HOMA-IR	3.1 ± 1.0	3.1 ± 1.1	3.4 ± 1.5	3.4 ± 1.3	2.7 ± 1.1	2.9 ± 1.1	0.82
GPx (μmol/L)	393 ± 44	399 ± 44	396 ± 43	400 ± 43	435 ± 35	439 ± 33 *	0.75
SOD (ng/mL)	20 ± 3	20 ± 2	21 ± 3	20 ± 2	21 ± 4	20 ± 3	0.42
Ox-LDL (U/L)	65 ± 13	65 ± 14	69 ± 12	68 ± 12	72 ± 12	72 ± 12	0.53
MDA (μm/L)	1.0 ± 0.37	1.1 ± 0.39	0.94 ± 0.39	0.99 ± 0.38	1.1 ± 0.45	1.0 ± 0.38	0.76

Data are the mean ± SD. *p*-values of ANOVA time-by-group interaction. Abbreviations: ADMA, asymmetric dimethylarginine; HOMA-IR, homeostatic model assessment for insulin resistance; GPx, glutathione peroxidase; SOD, superoxide dismutase; Ox-LDL, oxidized LDL; MDA, malondialdehyde. * $p < 0.05$ vs. before; ‡ $p < 0.01$ vs. before; † $p < 0.05$ vs. placebo and CIT+GSH; # $p < 0.05$ vs. CIT+GSH.

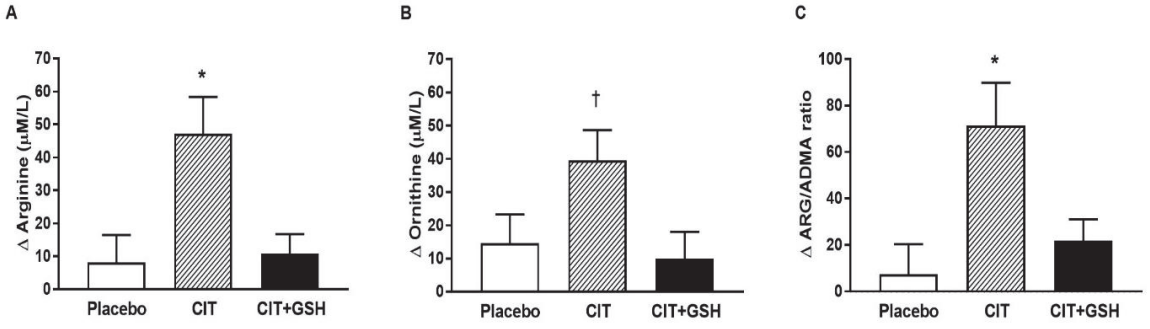

Figure 5. Changes (Δ) in serum levels of arginine (**A**), ornithine (**B**), and the arginine/ADMA ratio (**C**) from 0–4 weeks in the three groups. Values are the mean ± SE. Abbreviations: ARG, arginine; ADMA, asymmetric dimethylarginine; CIT, citrulline; CIT+GSH, CIT+glutathione. * $p < 0.05$ vs. placebo and CIT+GSH; † $p < 0.05$ vs. CIT+GSH.

4. Discussion

This study examined the effects of 4 weeks of CIT and CIT+GSH supplementation on vascular function and BP responsiveness in healthy postmenopausal women. We found that CIT+GSH supplementation improved endothelial function through an increase in the ARG/ADMA ratio. Although CIT supplementation increased serum ARG levels and the ARG/ADMA ratio, it did not statistically improve FMD. In addition, CIT+GSH supplementation attenuated BP responsiveness to CPT. These data show that CIT+GSH supplementation has protective cardiovascular effects at rest and during sympathetic stimulation (CPT) in healthy postmenopausal women.

FMD decreases progressively throughout the stages of the menopausal transition in healthy women [4]. Low FMD is a valuable biomarker for predicting future cardiovascular events in apparently healthy adults [39]. In this study, we showed that 4 weeks of CIT+GSH supplementation increased brachial FMD by 2.9% in healthy postmenopausal women. Consistent with our findings, a non-placebo controlled study observed a beneficial effect of CIT supplementation for 4 weeks on FMD in middle-aged patients with vasospastic angina and low FMD [40]. Similarly, 2 weeks of ARG supplementation increased FMD by 3.1% in healthy older men with age-related endothelial dysfunction [21]. A previous meta-analysis suggested that ARG supplementation improves FMD in individuals with baseline FMD values < 7.0% [41]. These previous findings suggest that CIT and ARG improve FMD in individuals with low FMD. Our data suggest that improvements in FMD following CIT+GSH mainly occurred in women with a low baseline FMD. Importantly, we found that the increase in FMD after 4 weeks of CIT+GSH was 0.91 SD greater vs. placebo and 0.67 SD greater vs. CIT alone. Given that for each 1 SD increase in FMD there is an associated 50% lower risk of cardiovascular events [42], our findings are clinically important.

Recent data showed that FMD values lower than 5.4% are indicative of impaired endothelial function in apparently healthy adults [43]. Based on this FMD reference value, 85% of women in the CIT+GSH group had low FMD, and thus, had impaired endothelial function at baseline. Evidence indicates that cardiometabolic risk factors, including BP and FBG, are determinants of low FMD [43]. Independent of traditional cardiovascular risk factors, low FMD predicts CVD risk [44]. In our study, an elevated baseline FBG was observed in the CIT+GSH group. Based on FBG values in the CIT+GSH group, four and seven participants had prediabetes or an increased risk of incident prediabetes, respectively [45]. It is known that prediabetes can negatively impact the age-related decline in FMD [46,47]. We demonstrate that CIT+GSH supplementation increased the mean FMD above 5.4% in a group of postmenopausal women with elevated FBG. Considering that a 4.3% increase in FMD is associated with 50% CVD risk reduction [42], the 2.8%

improvement in FMD following 4 weeks of CIT+GSH may reduce the risk of cardiovascular events in apparently healthy postmenopausal women.

Age associated arterial stiffening affects the aorta to a greater extent than peripheral muscular arteries [48]. In older women, 10 years of aging have a greater impact on aortic PWV (+2.4 m/s) than brachial PWV (+0.19 m/s), indicating that peripheral arteries have less stiffening than the aorta [48]. A widely used measure of arterial stiffness is brachial-ankle PWV (baPWV), a segment that includes central (aortic PWV) and peripheral (brachial and leg PWV) arteries [49]. Since peripheral arteries have relatively more smooth muscle cells than collagen fibers [48], brachial PWV and leg PWV are more responsive to NO-mediated vasodilation than the aorta [50,51]. Oral CIT supplementation (5.6 g daily) for 1 week significantly reduced baPWV by ~0.14 m/s in middle-aged men with high baseline baPWV [27]. A further study clarified that the beneficial effect of CIT (6 g daily) on baPWV was due to a reduction of leg PWV (~0.40 m/s) with no effect on aortic PWV in obese postmenopausal women [29]. In the present study, aortic PWV was not reduced by CIT and CIT+GSH supplementations. This ineffectiveness may be attributed to the normal baseline values of our participants, which are considered to be ~7.6 m/s for adults aged ≥50 years [38]. Importantly, CIT+GSH decreased brachial PWV by ~0.40 m/s in postmenopausal women with increased risk of prediabetes. This finding is in agreement with previous studies [27,29] and confirms that peripheral arteries are more responsive than the aorta to dietary supplementation with NO precursors. The decrease in brachial PWV observed in the current study may be attributed to enhanced endothelial-mediated vasodilation [51].

Impaired FMD and increased sympathetic activity are associated with the increased risk of incident hypertension in healthy postmenopausal women [13,14]. Our participants had a normal or elevated SBP at rest. Our observation that the resting BP was unaffected by CIT and CIT+GSH supplementations is consistent with previous studies in normotensive adults [30,32] and middle-aged adults with elevated SBP and FBG [27]. Thus, we used the CPT as a systemic sympathetic vasoconstrictor stimulus to evaluate the efficacy of dietary supplements for attenuating BP reactivity to CPT [18,19]. We observed reductions of brachial and aortic SBP and MAP responses to CPT after CIT+GSH but not CIT supplementation. Previous evidence of blunted BP responsiveness to CPT after 2 weeks of CIT (6 g/day) was observed in healthy young [31] and older adults [32]. However, we did not measure FMD in those previous studies. In mice, CIT supplementation attenuated cold hypersensitivity by improving endothelial function [52]. In the current study, the attenuation of aortic MAP reactivity to CPT was related to improvements in FMD. This finding suggests that improved FMD with CIT+GSH supplementation may reduce the risk of cardiovascular events related to the augmented aortic BP load during conditions with increased sympathetic stimulation [16,19,53].

ADMA competes with ARG for binding to eNOS, thereby a decrease in ARG/ADMA leads to reduced NO production [54]. Evidence supports that the ARG/ADMA ratio is a better biomarker of endothelial function than circulating ARG and ADMA levels alone [7,55]. Our participants had normal ARG/ADMA values at baseline [56]. However, the CIT+GSH group tended to have a lower ARG/ADMA ratio, which has been associated with hyperglycemia [57]. In the present study, CIT and CIT+GSH supplementations increased the ARG/ADMA ratio, suggesting increased ARG availability for NO production [54]. This finding is consistent with Ochiai et al. [27] who reported an increase in plasma ARG/ADMA due to the isolated increase in ARG levels. CIT supplementation increases circulating ARG via de novo synthesis in the kidneys [54]. Serum ARG introduction to endothelial cells via the cationic amino acid y+ transporter 1 depends on both ARG and ADMA levels, since they compete for the transporter [58]. Therefore, a greater ARG availability displaces ADMA from eNOS binding, thereby improving NO synthesis [7,27,40,54,59]. A recent study reported that 2 g of CIT for 4 weeks increased NOx levels in type 2 diabetes patients as a result of arginase inhibition [23]. These findings suggest that a low dose of CIT may improve endothelial function in individuals with hyperglycemia. In the study by Ochiai et al.,

the increase in ARG/ADMA was evident after CIT supplementation in men with FBG in the prediabetes category [27]. Therefore, augmented ARG/ADMA ratio may explain the improvement in endothelial function following CIT and ARG supplementations in adults with hyperglycemia [27] and low baseline FMD [21,40,60].

Despite greater increases in the serum ARG levels and ARG/ADMA ratio, FMD did not improve after CIT supplementation alone. This discrepancy in the findings may be explained by the healthy status of the participants. In agreement with our findings, ARG supplementation for 4 weeks increased serum ARG levels by almost double but failed to improve FMD in healthy postmenopausal women [61]. Similarly, 6 g of CIT supplementation efficiently increased circulating ARG levels and ARG/ADMA without affecting FMD in healthy adults [24]. In the present study, the increase in ARG levels positively increased the ARG/ADMA ratio, indicating improved ARG availability for NO production. Similarly, previous studies failed to show improvement in endothelial function assessed as increased NOx after oral ARG and CIT supplementations in healthy adults [25,62]. Thus, increased ARG availability via CIT or ARG supplementation may not improve endothelial function in individuals with normal FMD [24,61]. Of note, CIT is concurrently produced with NO by eNOS, and recycled to de novo ARG [63]. It is possible that CIT supplementation stimulated the production of NO and CIT, but the effect on FMD was not evident in the absence of endothelial dysfunction. Nonetheless, the increase in the ARG/ADMA ratio by CIT may have vascular benefits, since an increase in this ratio by 1 SD may decrease CVD risk by 20% [64].

Oxidative stress markers were not improved by both supplementations. In healthy adults, 200–1000 mg of GSH daily for 4 weeks did not improve microvascular endothelial function and malondialdehyde levels [65,66]. Recently, whole blood GSH was elevated after 1 month with 250 or 1000 mg of GSH supplementation [67]. Despite no improvement in the oxidative stress markers, CIT+GSH increased FMD by improving eNOS function via increased ARG availability [33]. The ability of CIT+GSH to increase NO production was demonstrated in human umbilical vein endothelial cells after exposure for 24 h to the combination but not to CIT and GSH alone [34]. Thus, GSH provides an augmenting effect to the conversion of ARG to NO [34].

There are some limitations in the present study. The sample size was relatively small and included healthy postmenopausal women. FBG was not considered for randomization, which resulted to be higher in the CIT+GSH group. Future studies might investigate the effects of CIT and CIT+GSH in individuals with prediabetes. A GSH dose greater than 200 mg daily for longer than 4 weeks of supplementation may reduce markers of oxidative stress, as previously shown [67]. We measured serum levels of superoxide dismutase and glutathione peroxidase, two intracellular enzymes, rather than intracellular enzymatic activity. A statistical limitation could be a "regression to the mean" phenomenon when examining changes in FMD from baseline. However, there were no significant decreases in FMD in the placebo group after the 4-week intervention, which supports our conclusion of CIT+GSH being a viable route to improve endothelial function in healthy postmenopausal women.

5. Conclusions

Four weeks of CIT+GSH supplementation improves brachial artery FMD in apparently healthy postmenopausal women with slightly elevated FBG. CIT+GSH reduced brachial PWV and attenuates BP responses to sympathetic activation. These beneficial effects on the endothelial function and BP reactivity may be attributed to an increased ARG/ADMA ratio. Therefore, CIT+GSH supplementation may reduce the risk of cardiovascular events during physiological stress in postmenopausal women. Although CIT supplementation alone caused greater increases in the serum ARG levels and ARG/ADMA ratio, it did not improve FMD or BP responses to CPT in healthy postmenopausal women.

Author Contributions: A.F., M.M. and D.N. were responsible for the study design. A.F. was responsible for the conduction of the study. A.M., S.M.F., M.A.M., Y.K. and K.N.D. performed the experiment and data collection. A.F., A.M., S.M.F., M.A.M., Y.K. and K.N.D. participated in the data analysis and interpretation. A.F., M.A.M., Y.K. and K.N.D. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by Kirin Holdings Co., Ltd., grant A20-0164-001.

Institutional Review Board Statement: The experimental protocol and informed consent was approved by Texas Tech University Institutional Review Board.

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

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: The authors recognize the cooperation and enthusiasm of our participants.

Conflicts of Interest: M.M. and D.N. are employees of Kirin Holdings Co., Ltd. M.M., D.N. and Kirin had no influence on participant handling, data collection, analysis or interpretation, and manuscript writing. The other authors declare no conflict of interest.

Abbreviations

ADMA	Asymmetric dimethylarginine
ARG	L-Arginine
baPWV	Brachial-ankle pulse wave velocity
BP	Blood pressure
cdPWV	Carotid-distal pulse wave velocity
cfPWV	Carotid-femoral pulse wave velocity
CIT	L-citrulline
CPT	Cold pressor test
crPWV	Carotid-radial pulse wave velocity
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
eNOS	Endothelial nitric oxide synthase
faPWV	Femoral-ankle pulse wave velocity
FBG	Fasting blood glucose
FMD	Flow-mediated dilation
GSH	Glutathione
MAP	Mean arterial pressure
NO	Nitric oxide
NOx	NO metabolites
ORN	Ornithine
PWV	Pulse wave velocity
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error

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Postprandial Glycemic and Insulinemic Response by a Brewer's Spent Grain Extract-Based Food Supplement in Subjects with Slightly Impaired Glucose Tolerance: A Monocentric, Randomized, Cross-Over, Double-Blind, Placebo-Controlled Clinical Trial

Hammad Ullah [1], Cristina Esposito [1], Roberto Piccinocchi [2], Lorenza Francesca De Lellis [1], Cristina Santarcangelo [1], Alessandro Di Minno [1,3], Alessandra Baldi [1], Daniele Giuseppe Buccato [1], Ayesha Khan [4], Gaetano Piccinocchi [5], Roberto Sacchi [6] and Maria Daglia [1,7,*]

1 Department of Pharmacy, University of Naples Federico II, Via Domenico Montesano 49, 80131 Naples, Italy
2 Level 1 Medical Director Anaesthesia and Resuscitation A. U. O. Luigi Vanvitelli, Via Santa Maria di Costantinopoli, 80138 Naples, Italy
3 CEINGE-Biotecnologie Avanzate, Via Gaetano Salvatore 486, 80145 Naples, Italy
4 Department of Medicine, Combined Military Hospital Nowshera, Nowshera 24110, Pakistan
5 Comegen S.c.S., Societ'a Cooperativa Sociale di Medici di Medicina Generale, Viale Maria Bakunin 41, 80125 Naples, Italy
6 Applied Statistic Unit, Department of Earth and Environmental Sciences, University of Pavia, Viale Taramelli 24, 27100 Pavia, Italy
7 International Research Center for Food Nutrition and Safety, Jiangsu University, Zhenjiang 212013, China
* Correspondence: maria.daglia@unina.it

Citation: Ullah, H.; Esposito, C.; Piccinocchi, R.; De Lellis, L.F.; Santarcangelo, C.; Minno, A.D.; Baldi, A.; Buccato, D.G.; Khan, A.; Piccinocchi, G.; et al. Postprandial Glycemic and Insulinemic Response by a Brewer's Spent Grain Extract-Based Food Supplement in Subjects with Slightly Impaired Glucose Tolerance: A Monocentric, Randomized, Cross-Over, Double-Blind, Placebo-Controlled Clinical Trial. *Nutrients* **2022**, *14*, 3916. https://doi.org/10.3390/nu14193916

Academic Editors: Abeer M. Mahmoud and Maria G. Grammatikopoulou

Received: 10 August 2022
Accepted: 15 September 2022
Published: 21 September 2022

Abstract: Dietary fiber exerts beneficial effects on human health reducing the risk factors of metabolic related diseases such as hyperglycemia, insulin resistance, and hypercholesterolemia. The aim of this study is to demonstrate the efficacy of a food supplement based on brewer's spent grain (BSG) extract in the reduction of postprandial glycemia and insulinemia in normoglycemic subjects. BSG was chemically characterized, revealing the presence of resistant starch (14.64 g/100 g), arabinoxylans (7.50 g/100 g), β-glucans (1.92 g/100 g) and other soluble fibers (6.43 g/100 g), and bioaccessible ferulic acid (91.3 mg/100 g). For the clinical study, 40 normoglycemic subjects were randomized into two groups, 1 and 2 (n = 20), for a cross-over clinical design and received either BSG extract-based food supplement or placebo. Postprandial blood glucose values were significantly lower than corresponding values in the placebo group after 90 and 120 min, while at the baseline and in the first 60 min, the two glycemic curves overlapped substantially. This improved clinical outcome was corroborated by significant reductions in postprandial insulinemia. None of the subjects reported adverse effects. This study showed that the tested BSG extract-based food supplement improves glucose metabolism and insulinemic response in normoglycemic subjects with at most a mild insulin resistance.

Keywords: brewer's spent grains; clinical trial; dietary fiber; food supplement; insulinemia; postprandial glycemia

1. Introduction

A large body of evidence suggests that dietary fiber, especially soluble fiber, exerts beneficial effects on human health, as it may reduce the risk of cardiovascular diseases, metabolic related issues, including diabetes and obesity, gastrointestinal ailments, and cancer along with the improvement of mental health and other cognitive functions [1–4]. The effects of dietary fiber can be exerted both directly, through the reduction of lipid and glucose absorption, which in turn decreases blood lipids and maintains blood glucose levels, and indirectly through its prebiotic effect, which leads to the growth of eubiotic

bacteria (i.e., Blautia, Roseburia, and Turicibacter) producing short chain fatty acids (SCFAs, i.e., acetic acid, propionic acid, and butyric acid), which improve intestinal permeability, insulin sensitivity, and relieve inflammation and glucose intolerance [5].

Among the various dietary fiber categories, arabinoxylans (AXs) are non-starch polysaccharides composed of a central linear carbonaceous skeleton consisting of β-1,4-linked xylose monomers (D-xylopyranose) and, as substituents, arabinose (L-arabinofuranose) side chains, which may have a ferulic acid on the fifth carbon. The covalent bond between ferulic acid residues is primarily responsible for the formation of gels, which in turn are responsible for simple sugar and lipid absorption reduction [6]. The main sources of AXs are wheat, barley, rice, rye, oats, and sorghum [7]. AXs, based on their structural and conformational properties, are classified into water extractable and non-water extractable AXs. The ratio between the two types of compounds varies depending on the species, cultivar, type of caryopsis tissue (bran, endosperm), external environment, and processing or extraction process [8]. AXs cannot be degraded by mammalian enzymes present in the digestive tract but are degraded by the gut microbiota into SCFAs and other products [9]. In a recent study by Lynch et al., soluble arabinoxylan extracted from brewer's spent grains (BSG) showed prebiotic effects, resulting from 2-fold and 3.5-fold increases in *Lactobacillus* and bifidogenic levels, respectively [10]. AXs also possess antioxidant capacity, with higher antioxidant activity when ferulic acid is bound [11,12].

In recently published studies, water-soluble AXs from wheat have been found to be effective in modulating the metabolism of glucose. The mechanisms of action that contribute to reducing glucose absorption and attenuating the postprandial glycemic response involve delaying gastric emptying time, slowing intestinal transit, reducing the glucose diffusion rate in the intestinal lumen, lowering the availability and inhibiting the activity of digestive enzymes in the intestinal lumen, and the bifidogenic effect [13–16]. In addition, the gelling properties of AXs delay the degradation and, consequently, the absorption of proteins by attenuating the insulinemic response due to insulinogenic amino acids [17]. In view of such properties, wheat endosperm AXs have been granted a European health claim for reducing postprandial blood glucose [18].

The growing interest in food fiber and especially AXs has led research towards the development of new ingredients to be used in the production of functional foods and food supplements using by-products of the agri-food industry to develop products that are economically and environmentally sustainable. In keeping with this, as BSG is a by-product of the brewing industry rich in insoluble fiber (i.e., cellulose, hemicellulose, and lignin) and soluble dietary fibers, especially AXs, it is an ideal candidate to be exploited as a raw material from which to obtain extracts with a high fiber content [19]. Nowadays, 70% of BSG are used as animal feed, 10% are used for biogas production, and the remaining 20% are disposed of in landfills [20]. Considering that about 3.4 and 4.5 million tons of BSG are generated in Europe and the USA, respectively, and a medium-sized brewery produces about 7–8 tons of BSG/week, the enhancement of these by-products to obtain food ingredients rich in fiber for the food industry with high added value is another possible way to utilize BSG [21].

Thus, the aim of this study is the evaluation of the properties of reducing post-prandial glycemia and improving insulinemia response of a food supplement containing a BSG extract chemically characterized in terms of total fibers, AXs, β-glucans, resistant starch, total polyphenols, and ferulic acid in healthy subjects through a randomized, cross-over, double-blind, placebo-controlled clinical trial.

2. Materials and Methods

2.1. BSG Extract-Based Food Supplement, and Placebo Used in the Clinical Study

BSG extract-based food supplement and placebo were produced by HEALLO s.r.l. (Milan, Italy), within European specifications for contaminants and microbiologic limits. The food supplement has been notified to the Italian Health Ministry, with the brand name "JAX Plus®" (notification number: 141039). The BSG extract-based food supplement, in the

form of soluble granules in single-dose stick packs (5 g), contains 5.0 g/stick pack of BSG extract, which consist of 4.25 g of BSG extract and 0.75 g (corresponding to 15%) of inulin used as carrier agent. Placebo in the same form consisted of microcrystalline cellulose and the same amounts of inulin (15%). The bakery product (breadsticks) consumed by the subjects recruited in the present clinical study was previously portioned and packaged (net weigh 65 g) in such a quantity as to provide 50 g of available carbohydrates and was characterized in terms of nutrients and caloric value (Table 1).

Table 1. Breadsticks nutritional values.

Average Nutritional Values	g/100 g of Product
Energy	1601 kJ–378 kcal
Fats	2.5 g
of which saturated fatty acids	0.5 g
Carbohydrates	74.5 g
of which sugars	3.4 g
Dietary fibers	4.8 g
Protein	12.7 g
Salts	1.3 g

2.2. Chemical Characterization of BSG Extract

2.2.1. Total Dietary Fiber Determination

Total dietary fiber content (TDF) was determined using the Total Dietary Fiber Assay Kit (Neogen, Lansing, MI, USA) according to the manufacturer's protocol [22], which represents a simplified version of the official AOAC 985.29 method for the determination of total fiber. TDF was determined on quintuplicate samples. Five aliquots of BSG extract (1.000 ± 0.005 g) were incubated at about 100 °C with 50 µL heat-stable α-amylase solution to allow starch gelatinization, hydrolysis, and depolymerization. The samples were then incubated at 60 °C with 100 µL protease solution (to solubilize and depolymerize the proteins) and 200 µL amyloglucosidase solution (to hydrolyze the starch fragments into glucose). The samples were filtered to separate the insoluble fiber. The supernatants were then treated with approximately four volumes of ethanol to allow precipitation of the soluble fibers and remove the depolymerized proteins and glucose (from starch). The samples were then filtered and the residues, corresponding to the soluble fiber, were washed with 78% ethanol, 95% ethanol, and acetone, and dried overnight in a microwave oven at 103 °C and then weighed. The first residue was analyzed for proteins, determined using the Bradford method [23], and the second one was incubated at 525 °C to determine the ash content. TDF was calculated as the sum of the weight of insoluble fiber and soluble fiber dry residue minus protein and ash weights.

The other three residues were stored at room temperature pending analysis to determine the content of AXs, β-glucans, and resistant starch.

2.2.2. Glucose, Arabinose, Xylose, Total B-Glucans, Total Arabinoxylans, and Resistant Starch Determination

According to the manufacturer's protocol, the BSG extract and the three soluble fiber dry residues previously described, obtained in the total dietary fiber assay, were prepared for carrying out the determination of (1) free glucose, (2) glucose deriving from rapidly digestible starch and slowly digestible starch, (3) glucose from resistant starch, (4) arabinose, (5) xylose, (6) AXs, and (7) β-glucans using the commercially available kits for the measurements of xylose, arabinose, glucose, and mixed linkage β-glucan (Neogen, Lansing, MI, USA).

As far as the BSG extract is concerned, glucose, arabinose, and xylose, present in the extract before hydrolysis, were quantified by spectrophotometric analysis [24,25], using three calibration curves prepared with standard compounds at known concentrations. The results of these analyses provided the content of free simple sugars originally present in BSG

extract. Then, BSG extract was submitted to hydrolysis with α-amylase and α-glucosidase at 60 °C for 30 min and for 4 h to obtain the content of glucose derived from rapidly digestible starch and slowly digestible starch and from resistant starch, respectively. Subsequently, glucose, and arabinose and xylose present in the extract after the hydrolysis of β-glucans and AXs, respectively, were quantified by spectrophotometric analysis. BSG extract β-glucan and AX concentrations were calculated on the basis of glucose, and arabinose and xylose concentrations, minus free glucose, and free arabinose and xylose, originally present in the extract before the enzymatic hydrolysis.

With regards to the soluble fiber dry residues obtained from the total fiber assay, glucose, and arabinose and xylose, present in the dry residue after the hydrolysis of β-glucans, AXs, and resistant starch, respectively, were quantified by spectrophotometric analysis, and β-glucan, AX, and resistant starch concentrations were calculated on the basis of glucose, and arabinose and xylose concentrations.

2.2.3. Water and Alkali Extractable Arabinoxylans

The isolation of the water extractable arabinoxylans (WEAX) and alkali extractable arabinoxylans (AEAX) in BSG extract was performed using a method from Buksa et al. [26]. After isolation, the quantification of WEAX and AEAX was performed according to the method reported in Section 2.2.2.

2.2.4. Total Polyphenol Content and Ferulic Acid Determinations

Simulated In Vitro Oral-Gastric-Duodenal Digestion Process of BSG Extract

The simulated in vitro oral-gastric-duodenal digestion of BSG extract was performed following the protocol by Minekus et al. with some modifications [27]. The used reagents are reported below: potassium chloride (KCl), dihydrogen potassium phosphate (KH_2PO_4), sodium carbonate ($NaHCO_3$), magnesium chloride ($MgCl_2$), ammonium carbonate ($(NH_4)CO_3$, calcium chloride ($CaCl_2$), sodium chloride (NaCl), hydrochloric acid (HCl), and sodium hydroxide (NaOH). All were provided by Carlo Erba (Milan, Italy). Pancreatin from porcine pancreas (extract of pig bile), α-amylase from *Bacillus licheniformis*, pepsin from porcine gastric mucosa and porcine bile extract, formic acid solution (1 M), water, methanol, and acetonitrile LC–MS grade were sourced from Sigma-Aldrich, Merck KGaA (Milan, Italy).

In brief, 5 g of the BSG extract were dissolved in 3.5 mL of previously prepared simulated salivary fluid (SSF). Then, 0.5 mL (1500 U/mL) of fresh α-amylase solution were added to both samples. In the end, water was added to reach a final volume of 10 mL and the sample was incubated for 2 min at 37 °C. The bolus obtained in the previous phase was mixed with 7.5 mL of simulated gastric fluid (SGF) and 1.6 mL (25,000 U/mL) of fresh pepsin; the pH was then adjusted to 2.00 ± 0.02 using 1 M HCl. The sample was brought up to 20 mL volume and the mixture was incubated at 37 °C for 2 h in a shaking water bath. Subsequently, gastric chyme was incubated with 5 mL of fresh pancreatin (800 U/mL) and 2.5 mL of fresh bile mixture (160 mM) to reach a final volume of 32.5 mL. The sample was finally made up to a 40 mL final volume; the pH was adjusted to 7.00 ± 0.02 using 1 M NaOH and incubated at 37 °C for 2 h. At the end of the digestion process, the oral-gastric-duodenal digested sample was freeze dried and maintained at 4 °C pending total polyphenol content determination and RP-HUPLC-MS analysis.

Total Polyphenol Content

Total polyphenol content (TPC) of the BSG extract and the freeze-dried digested samples (1 mg) was determined using a Folin–Ciocalteu method (colorimetric assay), with slight modifications [28]. A 10 µL aliquot of one of the sample solutions, namely the BSG extract, digested BSG extract (50 mg/mL), and gallic acid standard solutions (at concentrations ranging from 200 to 1000 µg/mL), was added to 50 µL of Folin–Ciocalteu reagent. The solutions were cyclomixed for 4 min, followed by the addition of 15% Na_2CO_3 (200 µL). Distilled water was added to the mixture to make the final volume up to 1 mL, and then allowed to incubate at room temperature for 2 h. The absorbance of the mixtures

was read at the wavelength of 750 nm and the results were expressed as mg equivalent to gallic acid/g (GAE/g) of the extract on a dry weight basis.

UHPLC-MS Analysis and Quantification of Ferulic Acid after Digestion

BSG extract freeze-dried digested samples (1 mg) was solubilized in 1 mL of water and treated with 3 mL of ice-cold acetonitrile. The sample was centrifuged for 15 min at 16,000 rpm (Hermle®, Hamburg, Germany), and the solvent was evaporated with nitrogen; the supernatant was solubilized in 50:50 methanol/water 0.1% formic acid and analyzed.

HUPLC-MS/MS analyses for the identification and quantification of ferulic acid in the digested sample were performed on a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Milan, Italy) consisting of two pumps, an autosampler, and a column oven. The system was coupled to a Linear Ion Trap Mass Spectrometer LTQ XL (Thermo Fisher Scientific, Milan, Italy) equipped with an electrospray source (ESI).

The separation was performed on a LUNA® OMEGA POLAR C18 column (L × ID) 150 mm × 2.1 mm, 3 µm (Phenomenex, Milan, Italy). The mobile phases used were A) 0.1% HCOOH in H_2O and B) ACN, with the following gradient: 0–5 min, 2% B; 5–15 min, 2–18% B; 15–30 min 18–95% B and hold for 5 min; returning to initial conditions in 0.2 min. The flow rate was set to 0.3 mL/min. Column oven was set to 45 °C, and 5 µL of extract were injected.

The ESI source was operated in the negative mode. MS/MS analyses were conducted in full mode, using the following ion with m/z 195.2 corresponding to ferulic acid, collision energy: 35.0 V, and scan range m/z 50–200. To optimize the MS operating conditions, a preliminary experiment was performed: 10 µg/mL ferulic acid (H_2O/MeOH: 50/50 with 0.1% formic acid) solutions were directly infused in the ESI interface at a flow rate of 25 µL/min into the mass spectrometer. Optimized conditions were as follows: sheath gas 60, capillary temperature 220 °C, auxiliary gas 25, spray voltage 4.5 kV, and capillary voltage −26.13 V for negative ionization mode. Ferulic acid (Sigma Aldrich, Milan, Italy) was selected as the external standard for the quantitation. Stock solution (1 mg/mL) was prepared in H_2O/MeOH: 50:50 with 0.1% formic acid, and the calibration curve ($y = 3669.9x - 50.911$) was obtained in the concentration range of 0.62–1 µg/mL ($R^2 = 0.9973$).

2.3. Clinical Trial Design

A monocentric, randomized, placebo-controlled, double-blind, cross-over clinical trial was performed by COMEGEN—Società Cooperativa Sociale (Naples, Italy) to evaluate the effects of the target food supplement based on BSG extract on the reduction of postprandial glycemic response in normoglycemic subjects. The study was double blind, both for the investigating physician and for the enrolled subjects. For this purpose, both the food supplement containing dietary fiber obtained from BSG extract and the placebo were made to be unrecognizable in shape, weight, color, and, as far as possible, in taste.

The participants received oral and written information regarding the study before they gave their written consent. Protocol, letter of intent of volunteers, and synoptic documents regarding the study were approved by the Scientific Ethics Committee of A.S.L. Napoli 1 CENTRO (Protocol number 222, 12 April 2021) and carried out in accordance with the Helsinki Declaration of 1964 (as revised in 2000). This study is listed on the ISRCTN registry (www.isrctn.com) with ID number 9301859. https://www.isrctn.com/ISRCTN19301859 (accessed on 5 August 2022).

The study design included two experimental groups (20 subjects for each group). The enrolled subjects were assigned to each of the two groups in a random and unpredictable way by means of a simple randomization (allocation ratio 1:1). At the baseline visit (t0), information on the sociodemographic, clinical, and biochemical characteristics (i.e., body mass index (BMI), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides) of the recruited subjects was collected and reported in the case report form (CRF). During the first visit (t0), the recruited subjects initially underwent a fasting blood draw. Then, the recruited subjects subsequently

consumed the standard meal, consisting of breadsticks eaten within 15 min with 500 mL of oligomineral water, and BSG extract-based food supplement (group 1) or the standard meal, oligomineral water, and placebo (group 2). Then, blood sampling at timed intervals measured postprandial glycemia and insulinemia (i.e., at 15 (t1), 30 (t2), 60 (t3), 90 (t4), and 120 (t5) min after the intake of breadsticks, water, and the treatment or placebo). This step was followed by a five-day wash-out period (in which the recruited subjects took no treatment), prior to the cross-over of treatments. After the five-day wash-out period, each subject in the two groups underwent blood sampling again (at the times indicated above) for measurement of blood glucose and insulin, before and after ingestion of the standard meal, oligomineral water, and placebo (group 1) or the standard meal, oligomineral water, and the food supplement (group 2), according to the cross-over design.

Participants were asked to reduce their fiber intake from two weeks before the start of the study until the end, and to not significantly change their eating habits for the entire duration of the study.

2.3.1. Study Population

Forty-subjects aged 18–65 years of either sex were recruited by the general practitioners of Comegen in September 2021. Inclusion criteria included healthy subjects (according to their clinical history), non-smokers, and subjects able to understand and to sign the informed consent. Subjects with type 1 or 2 diabetes, subjects with fasting blood glucose > 110 mg/dL, subjects with blood pressure values > 160/100 mmHg, subjects with metabolic or eating disorders, subjects with disorders that may have interfered with the results of the study (i.e., endocrine, cardiovascular, pulmonary, renal or gastrointestinal diseases), subjects sensitive, intolerant or allergic to the ingredients of the food supplement used in the clinical trial, pregnant or lactating women, blood donors in the three months prior to recruitment, subjects under pharmacological treatment with drugs that could interfere with the study (i.e., alpha-glucosidase inhibitors, insulin-sensitive drugs, sulfonylureas, cholesterol lowering drugs, and any other medications that the physician does not deem compatible with the study), subjects who were taking food supplements that could interfere with the study (i.e., products high in vitamins and minerals (>200% VNR), B vitamins, C vitamin, calcium, zinc, copper, chromium, iodine, iron, magnesium, manganese, phosphorus, essential fatty acid products, botanicals, and any other products that the physician does not deem compatible with the study) were excluded from the study.

2.3.2. Outcomes of the Study

The primary outcome of the present clinical study was to evaluate the contribution of the food supplement based on BSG extract, as part of a standard meal, in promoting the reduction of postprandial blood glucose increase in normoglycemic subjects. The secondary outcome was to evaluate the impact of the food supplement on the postprandial insulinemic response.

Data collection was performed by means of a CRF divided into two main sections. A first section concerning personal data, subject's medical history, intake of any concomitant drugs, and the treatment group, filled at the time of enrollment. The second section was filled with the results of the analyses performed on the blood samples taken.

2.3.3. Safety

BSG extract is an approved ingredient for food supplements. Although no adverse events related to the intake of the food supplement were expected, the enrolled subjects were continuously monitored for the occurrence of any kind of adverse effects. The subjects with sensitivity, intolerance, or allergy to gluten or barley were categorically excluded from the study.

2.4. Statistical Analysis

Sample size calculation was conducted using three 1-β power values (0.80, 0.95, and 0.99), a significance threshold value of α equal to 0.05, and three effect size values (Cohen's f = 0.20, 0.25, and 0.30, respectively). Sample size was determined to be 40 participants (20 each group).

The effect of the treatments on the response variables (blood glucose and insulin) was assessed through a linear mixed model (LMM), where the treatment groups (group A and group B), the measurement times (i.e., immediately after the meal, t0; after 15 min, t1; 30 min, t2; 60 min, t3; 90 min, t4; and 120 min, t5), the order of treatment (first and second) and the age and sex of the subjects were entered into the model as fixed effects. The measurement × treatment and measurement × treatment order interactions were included among the independent variables. The measure × treatment interaction is the key variable for the primary endpoint, as it allows testing whether the trend over the course of the measurements differs for the two treatments.

With regards to the interaction measurement × treatment order, it is used to check whether trends during the measurement period differ according to the order of administration of the treatments (before or after wash-out). The identity of the subject was evaluated as a random effect, which provided a control for differences among the enrolled subjects.

Analyses were performed using the lme4 [29] packages in R ver. 4.0.1 [30] (R Foundation for Statistical Computing, Vienna, Austria) and unless otherwise stated, data are reported as means ± standard errors.

For each subject of groups 1 and 2, the glucose and insulin incremental areas under the curves (iAUCs) were calculated. The iAUCs were evaluated statistically using a *t*-test: two paired samples for means, with each subject being his or her own control. Differences resulting in *p*-values below 0.05 were considered significant.

3. Results

3.1. Chemical Characterization of BSG Extract

In this study, the extract used was produced from brewer's spent grain.

First, the fiber present in the extract was characterized. To determine total soluble and insoluble fiber, the BSG extract was analysed by a gravimetric method involving the elimination of starch and proteins following treatment with α-amylase, amyloglucosidase, and protease, respectively. After this treatment, the BSG extract did not show any precipitate, indicating the absence of insoluble fiber. To determine the soluble fiber, 78% ethanol was added to the sample. The high molecular weight soluble fiber, precipitated and determined by a gravimetric method, resulted to be 7.45 g/100 g BSG extract (Table 2). Since low molecular weight soluble fiber does not precipitate into 78% ethanol, this fiber fraction was not determined with this assay. Therefore, total AXs and β-glucans were determined by hydrolyzing glycosidic bonds with specific enzymes and determining their concentrations on the bases of the concentrations of arabinose and xylose and glucose deriving from their hydrolysis, respectively. The results showed that total AXs and total β-glucans were 7.50 and 1.92 g/100 g of BSG extract, respectively. The same method was also applied to the total fiber dry residue isolated with precipitation under 78% ethanol to determine the content of high molecular weight AXs and β-glucans isolated by the above method. The concentration of AXs was 0.45 g/100 g, but β-glucans were not found to be detectable in the total fiber dry residue. The concentration of low molecular weight AXs soluble in 78% ethanol, and therefore not calculated with the gravimetric method, was calculated by the difference, and resulted to be 7.05 g/100 g of BSG extract.

Table 2. Concentrations of free glucose, arabinose, and xylose, total AXs, WEAX and AEAX, total β-glucans, glucose from rapidly and slowly digestible starch, and glucose from resistant starch, in BSG extract, and AXs, β-glucans, and resistant starch determined in the total fiber dry residue.

Compound	Concentration (g/100 g) [1]
Free glucose occurring in BSG extract	5.53 ± 0.01
Free arabinose occurring in BSG extract	0.71 ± 0.01
Free xylose occurring in BSG extract	0.95 ± 0.01
Total AXs occurring in BSG extract	7.50 ± 0.05
WEAX—water extractable arabinoxylans	1.23 ± 0.02
AEAX—alkali extractable arabinoxylans	6.36 ± 0.03
AXs insoluble in ethanol (78%) occurring in total fiber dry residue	0.45 ± 0.01
AXs soluble in ethanol (78%) [2]	7.05 ± 0.01
Total β-glucans occurring in BSG extract	1.92 ± 0.05
β-glucans insoluble in ethanol (78%) occurring in total fiber dry residue	N.D.[3]
Glucose after 30 min of enzymatic hydrolysis occurring in BSG extract [4]	30.36 ± 0.01
Glucose after 4 h of enzymatic hydrolysis occurring in BSG extract [5]	45.00 ± 0.06
Glucose after 4 h of enzymatic hydrolysis occurring in total fiber dry residue	0.62 ± 0.01
Total dietary fiber	7.45 ± 0.03

[1] Data expressed as means ± SD (n = 3). [2] calculated by the difference between total AXs present in the BSG extract and AXs present in the total fiber dry residue. [3] N.D. not detectable. [4] corresponding to glucose from rapidly and slowly digestible starch, including free glucose. [5] corresponding to glucose from resistant starch, including glucose from rapidly and slowly digestible starch and free glucose.

Gelling properties of AXs are mainly attributed to WEAX, with AEAX showing less gelling and therefore being less active in the modulation of glucose absorption. WEAX and AEAX were determined to have concentrations of 1.23 g/100 g (representing about 16% of total AXs) and 6.36 g/100 g (representing about 84% of total AXs), respectively.

Finally, the concentrations of (1) free glucose, (2) glucose derived from rapidly digestible starch and slowly digestible starch (including free glucose), and (3) total glucose deriving from resistant starch (including glucose derived from rapidly digestible starch, slowly digestible starch, and free glucose) were determined in the BSG extract. The concentration of glucose deriving from resistant starch, calculated by the difference between total glucose concentration determined after 4 h of enzymatic treatment, and after 30 min of enzymatic hydrolysis (corresponding to glucose concentration from rapidly digestible, slowly digestible starch, and free glucose), resulted to be 14.64 g/100 g of BSG extract. In addition, total glucose derived from resistant starch was isolated from the total fiber dry residue and resulted to be 0.62 g/100 g of BSG extract. Thus, the concentration of resistant starch soluble in 78% ethanol, and therefore not calculated with the gravimetric method, was calculated by the difference, and resulted to be 14.02 g/100 g of BSG extract.

In total, the whole BSG extract contained 30.40 g of dietary fiber/100 g of BSG extract, mainly represented by resistant starch (14.64 g/100 g), AXs (7.50 g/100 g), β-glucans (1.92 g/100 g), and other soluble fibers (6.38 g/100 g) isolated with 78% ethanol precipitation and calculated by the difference between total soluble fiber dry residue (7.45 g/100 g) and high molecular weight AXs (0.45 g/100 g) and resistant starch (0.62 g/100 g) weights.

TPC before and after oral-gastric-duodenal digestion were found to be 0.499 ± 0.01 and 1.16 ± 0.03 g GAE/100 g of BSG extract, respectively, suggesting that during digestion polyphenols are released by the food matrix and become bioaccessible. As spectrophotometric methods generally have issues with overestimating the phenolic contents, since Folin–Ciocalteu reagent interacts with non-polyphenolic molecules (i.e., reducing sugars), the ferulic acid content, which is the polyphenol most represented in BSG, was evaluated by means of a validated UHPLC-MS/MS method after oral-gastric-duodenal digestion [31]. Its identification was based on the mass spectrum and fragmentation pattern of the parent ion with m/z 193 (Figure 1). The content of ferulic acid was 64.8 ± 0.06 mg/100 g of digested BSG extract, corresponding to 91.3 ± 0.07 mg/100 g of BSG extract.

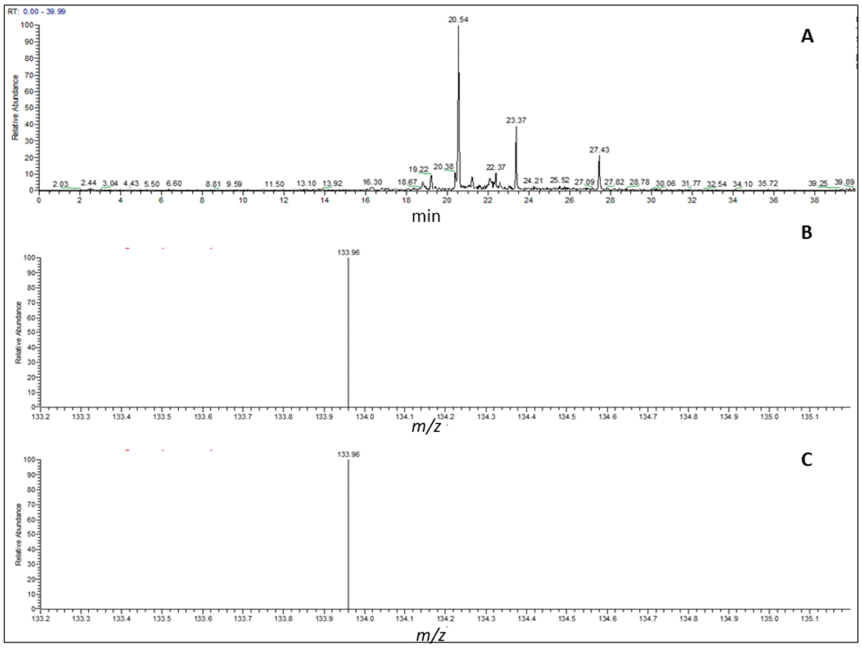

Figure 1. The chromatogram of an ion product with m/z 193 obtained from the HUPLC-MS/MS analysis of digested BSG extract (**A**). Chromatogram recorded at 320 nm; (**B**). Mass spectrum of parent ion with *m/z* 193; (**C**). Mass spectrum of fragmentation of parent ion with *m/z* 193. LOQ and LOD values determined for ferulic acid were 0.062 and 0.016 μg/mL, respectively.

3.2. Clinical Trial

The study flow chart, produced in accordance with the CONSORT PRO reporting guidelines [32], is shown in Figure 2. Initially, 42 subjects were screened for the clinical study; however, two of these subjects did not meet the inclusion criteria and therefore were excluded. The total number of subjects enrolled was 40, and they were randomly assigned to either group 1, receiving the BSG extract-based food supplement (treatment A) first and then the placebo (treatment B), or to group 2, which first received the placebo and then the BSG extract-based food supplement. As the aim of the clinical trial is to unravel the efficacy of BSG extract in reducing post-prandial glycemia and improving insulinemic response, the BSG extract-based food supplement was compared with the placebo consisting of indigestible carbohydrates. Group 1 consisted of 13 women (65%) and 7 men (35%), and group 2 consisted of 12 women (60%) and 8 men (40%).

The participants in the two groups had similar sociodemographic characteristics and clinical data, with no significant differences with the exception of HDL-C ($p = 0.01$). The baseline characteristics of the subjects for each group are summarized in Table 3.

The primary objective of the study was to evaluate the contribution of BSG extract-based food supplement in promoting the reduction of postprandial blood glucose in normoglycemic subjects with slightly impaired glucose tolerance, shown by HOMA-IR Index and Triglycerides and Glucose index (TyG) values higher than 2.5 and 4.5, respectively, for most of the recruited subjects [33] (Table 3). In fact, the upper limits of the ranges for triglycerides, HDL-C, and LDL-C greater than 150 mg/dL), lower than 40 mg/dL, and higher than 159 mg/dL, respectively, lead to thinking that part of the subjects recruited has an altered lipid profile compatible with mild insulin resistance [34] (Table 3). Nevertheless, at the baseline (t0) all 40 subjects were normoglycemic (Table 4).

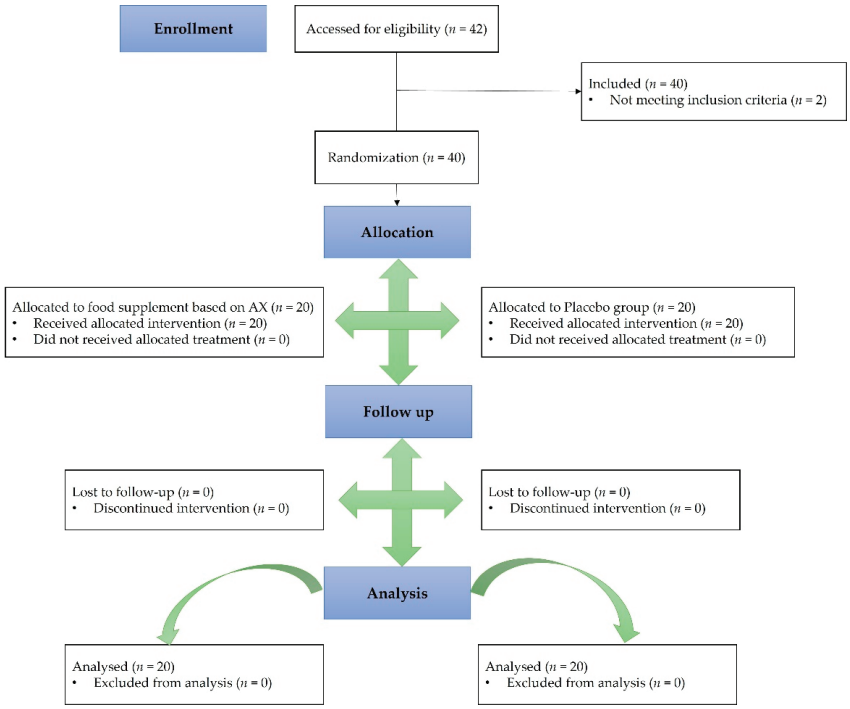

Figure 2. CONSORT Flow diagram.

Table 3. Characteristics of the study population: demographic and clinical data at baseline (t0).

Features	Group 1 (*n* = 20)	Group 2 (*n* = 20)
Age (years)	53 ± 5	57 ± 7
Gender:		
Male	7	8
Female	13	12
Ethnicity: European	20	20
BMI (kg/m^2)	21.82 ± 2.05 − (18.6–24.7)	21.93 ± 2.32 − (18.5–24.8)
TC (mg/dL)	217.25 ± 17.89 − (180–245)	220.7 ± 17.71 − (180–246)
HDL-C (mg/dL)	48.55 ± 12.14 − (30–65)	54.9 ± 9.18 − (33–68)
LDL-C (mg/dL)	112.85 ± 17.78 − (83–153)	120.25 ± 23.42 − (82–160)
Triglyceride (mg/dL)	131.55 ± 26.49 − (82–167)	125.6 ± 22.6 − (85–170)
Homa index	3.39 ± 1.32 − (1.21–5.25)	4.58 ± 1.45 − (1.54–6.81)
TyG index	8.51 ± 0.24 (7.99–8.86)	8.61 ± 0.21 (8.19–8.99)

Glycemia and insulin levels for the two study groups, at different time points, both in male and in female subjects, are reported in Table 4. As expected, the mean postprandial glycemia values recorded after 15 and 30 min tended to grow to a peak at 60 min, regardless of sex and treatment. After peaking at 60 min, the mean postprandial glycemia values recorded after 90 and 120 min tended to decrease more in the subjects who had taken the food supplement than those subjects who had taken the placebo, regardless of sex. In fact, while from 0 to 60 min the post-prandial glycemia values of the subjects (both male and female) who had taken the food supplement and the placebo were overlapping, at 90 and 120 min, the mean postprandial glycemia values of the subjects who had taken the food supplement were lower than the corresponding values of the subjects who had taken the placebo. As far as postprandial blood insulin is concerned, the mean post-prandial

insulinemia values recorded after 15 and 30 min tended to grow to a peak of 60 min in the placebo group more than in the food supplement group, regardless of sex. After the peak recorded at 60 min, also in this case the average post-prandial insulinemia values recorded after 90 and 120 min tended to decrease more in the subjects who had taken the food supplement than in those who had taken the placebo, regardless of gender.

Table 4. Variation in values (mean ± standard deviation, minimum and maximum) of blood glucose and insulin in men and women for the two experimental treatments (A: BSG extract-based food supplement, and B: placebo).

Variable / **Treatment**		**t0**	**t1**	**t2**	**t3**	**t4**	**t5**
Blood glycemia (mg/dL)							
Female	A	81.6 ± 7.7 (70–95)	86.9 ± 7.6 (75–100)	92.2 ± 7.8 (80–105)	97.5 ± 7.6 (85–110)	91.4 ± 7.3 (80–106)	83.3 ± 8.3 (72–101)
	B	81.5 ± 6.5 (70–94)	86.7 ± 6.4 (76–99)	91.8 ± 6.5 (81–104)	97.3 ± 6.6 (87–109)	93.2 ± 6.7 (82–105)	89.3 ± 6.5 (79–101)
Male	A	86 ± 8.1 (72–95)	91.3 ± 7.9 (77–100)	96.4 ± 7.8 (83–106)	101.4 ± 7.4 (88–110)	94.8 ± 8.1 (82–105)	88.3 ± 8 (74–96)
	B	84.3 ± 7.6 (74–94)	89.7 ± 7.4 (80–100)	95.3 ± 7.1 (86–105)	100.5 ± 6.7 (91–109)	96.5 ± 6.6 (85–104)	93.2 ± 6 (83–100)
Blood insulin (µU/mL)							
Female	A	17.3 ± 7.2 (6–30)	27.7 ± 7.2 (17–41)	37.8 ± 7 (27–52)	48.3 ± 7 (38–62)	30.5 ± 9.6 (18–48)	18.5 ± 7.1 (8–34)
	B	17.9 ± 8.1 (6–30)	30.2 ± 7.5 (20–41)	40.6 ± 7.7 (28–53)	51.3 ± 8.5 (34–64)	38.1 ± 7.7 (25–51)	26.8 ± 8.8 (9–39)
Male	A	16.3 ± 6.4 (7–28)	26.9 ± 6.2 (18–39)	36.9 ± 6.5 (28–49)	47.4 ± 6.1 (39–58)	30.3 ± 6.8 (20–42)	17 ± 5.4 (9–27)
	B	21.6 ± 6 (10–30)	33.7 ± 6.7 (21–45)	44.8 ± 6.9 (32–58)	55.2 ± 7.9 (43–72)	43.2 ± 5.7 (33–51)	30.9 ± 5.8 (20–40)

The LMM model for blood glucose (Table 5) identified a statistically significant effect for the measurement ($p < 0.001$), for the treatment ($p < 0.001$), and also for the measurement × treatment interaction ($p < 0.001$). Significant effects also emerged for sex ($p = 0.014$) and age ($p = 0.032$). Furthermore, the effect of the treatment order was also significant ($p < 0.001$); the measurement × order of treatment was not ($p = 0.99$). These results indicate that there was a difference between treatments A and B when it came to the postprandial glycemic curve of patients (Figure 3—top figure). Blood glucose did not differ between treatments, from the initial measurement to the peak at 60 min (t0: dB-A = 0.29 ± 1.21, t424 = 0.240, $p = 0.81$; t15: dB-A = 0.22 ± 1.21, t424 = 0.185, $p = 0.85$; t30: dB-A = 0.34 ± 1.21, t424 = 0.283, $p = 0.77$; t60: dB-A = 0.46 ± 1.21, t424 = 0.383, $p = 0.70$). Actually, the blood glucose values of the subjects treated with the food supplement (treatment A) or placebo (treatment B), recorded at t0, t1, t2, and t3, did not change. Differently, the blood glucose values recorded at t4 (90 min) and t5 (120 min) during treatment A were significantly lower than the corresponding values during treatment B in the subsequent phase of descent from the peak, respectively, after 90 min (dB-A = 2.67 ± 1.21, t424 = 2.199, $p = 0.028$) and 120 min (dB-A = 6.55 ± 1.21, t424 = 5.397, $p < 0.001$).

Table 5. LMM model results for glucose and insulinemic response curves.

Template	F	Df	P
Glycemia			
Measurement	89.11	5, 423	**<0.001**
Treatment	11.01	1, 427	**<0.001**
Sex	6.63	1, 37	**0.014**
Age	4.96	1, 37	**0.032**
Processing order	22.83	1, 423	**<0.001**
Measurement × Treatment	4.69	5, 423	**<0.001**
Measurement × Order of treatment	0.04	5, 423	0.99
Insulin			
Measurement	324.06	5, 423	**<0.001**
Treatment	138.95	1, 428	**<0.001**
Sex	1.36	1, 37	0.25
Age	2.58	1, 37	0.12
Processing order	6.87	1, 423	**0.0097**
Measurement × Treatment	6.29	5, 423	**<0.001**
Measurement × Order of treatment	1.86	5, 423	0.10

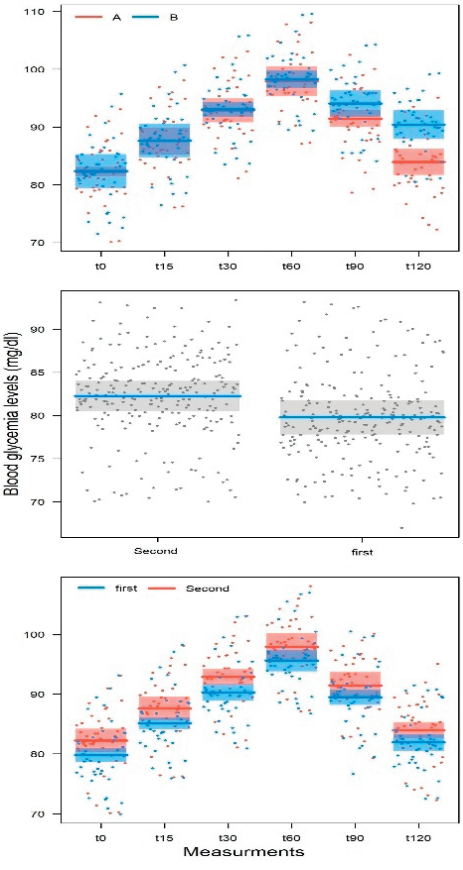

Figure 3. Variation in postprandial glycaemia for the two experimental treatments. **Above**: variation

in blood glucose for the two experimental treatments (A: BSG extract-based food supplement and B: placebo); **middle**: blood glucose values before and after wash out; **below**: change in blood glucose before and after wash out regardless of experimental treatment.

The trend of the glycemic curve, however, was not different before and after the washout period, as evidenced by the fact that the interaction between measurement and treatment order was not significant (Table 5) (Figure 3—middle and below figure).

The effect of sex indicates that the men selected in the sample had blood glucose values higher than those of women (4.12 ± 1.60, t36 = 2.575, $p = 0.014$). This difference, however, has no clinical relevance, as the blood glucose values of the subjects at t0 were in line with the inclusion criteria.

Finally, as far as the age of recruited subjects is concerned, blood glucose tended to increase with age (0.26 ± 0.11, t36 = 2.228, $p = 0.032$) regardless of gender. Moreover, in this case, this difference has no clinical relevance as the blood glucose values of the subjects at t0 were in line with the inclusion criteria.

The LMM model for insulin (Table 5) provided similar results to that for glycemia. The effects of measurement ($p < 0.001$), treatment ($p < 0.001$), and measurement × treatment interaction ($p < 0.001$) were statistically significant. There was no significant effect for the sex ($p = 0.25$) and age ($p = 0.12$).

The effect of the treatment order was also statistically significant in this case ($p = 0.009$), but the interaction between measurement and treatment order ($p = 0.10$) was not such. These results indicate that there is a difference in the postprandial insulin curve in patients undergoing treatment A versus those when undergoing treatment B (Figure 4—top figure).

The insulin values differed between treatments, since the initial measurement (dB-A = 2.83 ± 1.31, t424 = 2.169, $p = 0.031$) increased at 15 min (dB-A = 4.51 ± 1.31, t424 = 7.077, $p < 0.001$) and gradually at 30 min (dB-A = 5.02 ± 1.31, t424 = 7.590, $p < 0.001$), reached a peak at 60 min (dB-A = 5.10 ± 1.31, t424 = 7.650, $p < 0.001$) and 90 min (dB-A = 10.16 ± 1.31, t424 = 12.733, $p < 0.001$), with a maximum measurement at 120 min (dB-A = 10.79 ± 1.31, t424 = 13.336, $p < 0.001$).

This order indicates that the insulin value after the washout is significantly lower than that observed before the washout (−1.39 ± 0.53, t423 = 2.612, $p = 0.009$), regardless of the experimental treatment. As for glycaemia, the trend of the curve was not different between before and after the washout period for insulinemia, as evidenced by the fact that the measurement × treatment order was not significant (Table 5).

The incremental areas under curve (iAUCs) of postprandial glycemia of the subjects treated with BSG extract-based food supplement and the corresponding iAUCs of the subjects taking placebo did not show any statistical difference, while the mean incremental area under curve (iAUC) of insulinemia of the subjects treated with BSG extract-based food supplement (iAUC = 1928 ± 237) were 19.7% significantly lower (f-ratio value = 6.30397, p-value = 0.013436) than the corresponding mean iAUC of the subjects taking the placebo (iAUC = 2457 ± 400).

As far as safety is concerned, none of the subjects reported any adverse event after receiving the food supplement.

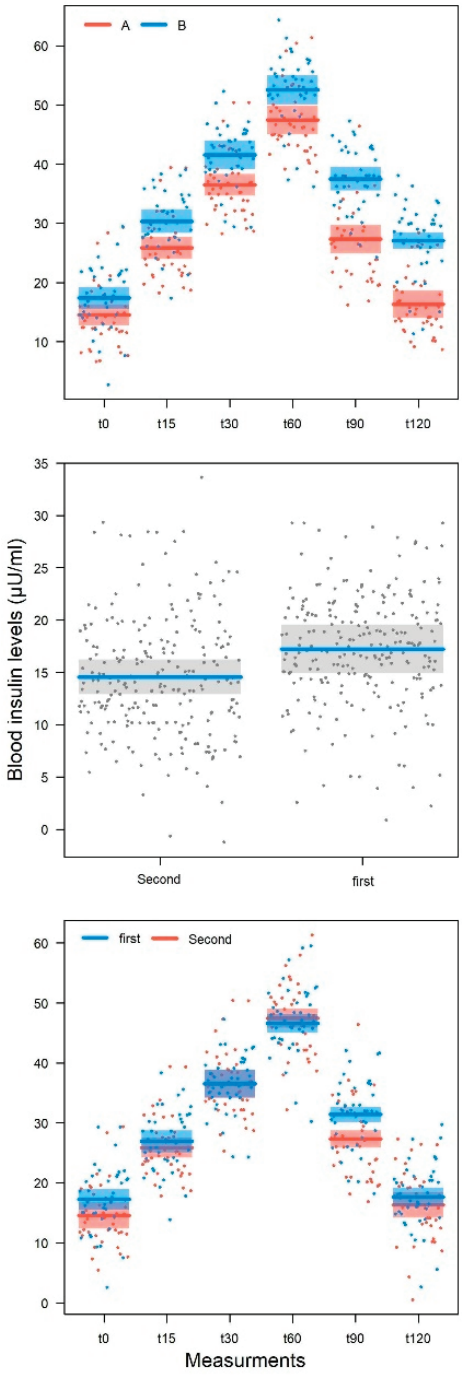

Figure 4. Variation in postprandial insulin values for the two experimental treatments. **Above**: variation of the insulin concentration for the two experimental treatments (A: BSG extract-based food supplement, and B: placebo); **middle**: insulin concentration values before and after washout; **bottom**: change in insulin concentration before and after washout regardless of experimental treatment.

4. Discussion

In this study, a new extract from brewer's spent grains was used as a bioactive ingredient for food supplements and tested for its effects on postprandial glycemia and insulinemic response in a monocentric, randomized, cross-over, double-blind, placebo-controlled clinical trial.

Although the literature data report that the main fiber families present in BSG are insoluble fibers (i.e., cellulose, lignin, and hemicellulose) [19], the BSG extract used in this study does not contain insoluble fiber. This composition is probably due to the patented enzymatic extraction method that allows enrichment of the extract with soluble fibers [35]. In agreement with the literature data [19], this BSG extract consists of resistant starch (14.6 g/100 g), followed by AXs (7.5 g/100 g) and β-glucans (1.9 g/100 g). Among AXs, 5% and 95% of the total AX content are represented by high molecular weight and low molecular weight AXs, respectively. Moreover, the water extractable AXs represent about 16% and alkali extractable AXs represent about 84% of total AX content. The major gelling properties, which, in turn, are responsible for the lower and slower absorption of glucose from the diet, are mainly ascribed to water-soluble, high molecular weight AXs. Based on the obtained results regarding AXs, we expect the BSG extract effect on glucose absorption to be modest.

On the basis of the results of the analyses of the different kind of fiber of BSG extract, which was found to contain about 31 g of soluble fiber per 100 g of BSG extract, one stick pack of the BSG extract-based food supplement, at 5.0 g/stick pack of BSG extract, contains about 1.5 g of soluble fibers and 0.75 g of inulin (about 7% of the recommend consumption of 25 g for adult women and 38 g for adult men, based on epidemiologic studies showing protection against cardiovascular disease) [36].

Most of the phenolic compounds present in barley grains are found in the husk, and thus BSG represents a rich source of polyphenols. The TPC obtained from the analysis of BSG extract before the in vitro simulated digestion process resulted to be 0.49 g GAE/100 g. This value is comparable to those reported by Birsan et al. that found that TPC of the crude extract ranged from 0.28 to 0.38 g GAE/100 g, and by Meneses et al. that reported that TPC of the BSG extracted with water resulted to be 0.39 g GAE/100 g [37,38]. After the in vitro simulated digestion process, TPC was found to be 1.16 g GAE/100 g. Although this result is an overestimation of polyphenol content, it is however indicative of the presence of bioaccessible polyphenols that are not degraded by digestion and that can perform their beneficial effects at the intestinal level.

Ferulic acid is the main free and bound polyphenol in barley, malt, and BSG [39]. As ferulic acid has been shown to exert anti-diabetic effects in many in vitro and in vivo studies through different mechanisms of action (i.e., reduction of oxidative stress in pancreatic islets, which, in turn, causes their necrosis leading to reduced secretion of insulin, improvement in the activities of antioxidant enzymes (i.e., superoxide dismutase and catalase) in the pancreatic tissue, and increase of glucose uptake in insulin resistant cells) [40], to understand how much ferulic acid remains available to exert its beneficial biological activities after digestion, the residual free ferulic acid content after in vitro simulated oral-gastric-duodenal digestion was determined. Ferulic acid concentration was found to be about 90 mg/100 g BSG extract. The ferulic acid content of BSG depends on the varieties of barley used, the malting and brewing processes, and the extraction method used to treat the sample before analysis. Mussatto et al. showed that by applying optimized alkaline hydrolysis conditions for the extraction of ferulic acid from BSG, its concentration resulted to be 9.65 mg/ per gram of solubilized lignin, corresponding to 286 mg/100 g of BSG [41]. The result obtained from the analysis performed in the present investigation showed a lower concentration of released ferulic acid in comparison with the result obtained by Mussatto et al. This result can be due to variations in the treatment of the samples that lead to different yields in the release of ferulic acid. Nevertheless, we decided to apply a simulated in vitro digestion process as it is more in line with the human digestive process and can give a more accurate view of the bioaccessibility and bioavailability of ferulic acid.

Regarding the results of the clinical trial on postprandial glycemia and insulinemia, a large body of evidence suggests that postprandial hyperglycemia is a risk factor for the onset of diabetes and cardiovascular disease [42,43].

The results of this clinical trial showed that blood glucose values measured after BSG extract-based food supplement intake were significantly lower than the corresponding values for the placebo group only in the descent phase of the glucose peak, respectively, after 90 min and 120 min, while at the baseline (t0) and in the first 60 min (t1–t3), the two glycemic curves overlapped substantially. We justify the absence of an effect of the intake of the food supplement taken with the standard meal on post-prandial blood glucose recorded in the first 60 min, with the low content and poor gelling properties of soluble fibers, especially AXs, present in BSG extract, that do not induce a significant reduction in glucose absorption. In addition, the glycemic curves of the subjects who took a placebo have trends typical of those curves recorded after the consumption of a starchy food. In fact, as reported by Brand-Miller et al. [44], starchy foods provide more glucose than sugary foods (i.e., soft drinks and fruit juices), and therefore were generally more likely to produce a curve that remained above baseline at 120 min. The intake of BSG extract-based food supplement, while not inhibiting the absorption of glucose, in the first 60 min, probably slightly reduces and slows down the absorption of glucose in accordance with the fiber content of the food supplement. The explanations of the modest recorded effect on postprandial glycemia produced by BSG extract based-food supplement may be due at least in part to the presence in the BSG extract of simple sugars, which increase glycemia, and to the fact that the effect of the food supplement is compared with the placebo that contains 15% inulin, which is a dietary fiber known to have hypoglycemic effects [45,46].

However, since the purpose of the clinical study was to test the effect of BSG extract, to exclude the effect of inulin used as carrier agent, the same amount of inulin present in the food supplement was added to the placebo. Although modest, the intake of the BSG extract based-food supplement produced a beneficial effect on glycemia as postprandial glycemic curve returned to baseline earlier. This improved clinical outcome was corroborated by significant reductions in postprandial insulinemic response. In particular, the blood insulin values of the subjects that took the food supplement were significantly lower from the first 15 min, with growing differences that reached maximum difference at 120 min from the first blood sample (t0). The mean insulin iAUC of the subjects who took the BSG extract-based food supplement was 19.7% significantly lower than the iAUC of the subjects taking placebo ($p < 0.05$). On the whole, these results mean that acute intake of BSG extract-based food supplement induces an improvement in postprandial insulinemic response. Moreover, it is worth noting that although the mean values of glycemia, BMI, and blood lipids of the recruited subjects are normal, however, on the basis of fasting glycemia, insulinemia, and triglyceridemia, most subjects showed a mild insulin resistance, as evidenced by HOMA Index and TyG values higher than 2.5 and 4.5, respectively. Thus, BSG extract-based food supplement, while not leading to a lowering of the postprandial glycemic curve, improved insulinemic response in subjects with mild insulin resistance.

Moreover, in accordance with the literature data, we found blood glucose values were significantly higher in men than in women irrespective of both the six measures the two experimental treatments, although these differences have no clinical relevance [47–50], and blood glucose tended to increase with age, independently from gender. It is well known that aging is associated with increased fasting blood glycemia due to a reduction in glucose-induced insulin release, and increased inflammation markers, which, in turn, increase insulin resistance in muscle and adipose tissue [51].

This work has limitations and strengths. The main limitations are represented by the fact that, due to the acute nature of this clinical trial, the effect of continuous ingestion of BSG extract and the influence of its fermentation products on glucose metabolism could not be determined, making it impossible to learn about any longer-term effects of this supplementation. Secondly, its effect on diabetic and pre-diabetic patients is unknown, as the subjects of this clinical trial were limited to normo-glycemic subjects showing just

mild insulin resistance. Finally, the presence in the food supplement of simple sugars, which together with the standard meal contribute to the rise of postprandial blood glucose, and the absence of an equal number of simple sugars in the placebo probably cause an underestimation of the actual properties of BSG extract-based food supplement to reduce the increase in post-prandial glycemia.

On the other hand, the major strength of this study is that the fiber and polyphenol composition of the BSG extract-based food supplement is known, and therefore the recorded effects on postprandial blood glucose and insulinemic response can be linked to the simultaneous presence of resistant starch, AXs, and β-glucans.

5. Conclusions

In conclusion, there is considerable importance in reducing postprandial glucose and insulin increases responsible for oxidative stress and β cells damage, which in turn are considered risk factors for serious chronic diseases. There is also a growing interest in the development of new economically and environmentally sustainable food products designed to improve glucose metabolism. The BSG extract-based food supplement tested, containing soluble dietary fiber and bioaccessible ferulic acid, was found to lead to the restoration at 120 min of blood glucose and insulin values to the values recorded at the baseline, reducing postprandial glucose and insulin increases in normo-glycemic subjects showing just a mild insulin resistance.

More studies are required to determine whether these acute benefits result in long-term improvements in glycemic control and whether supplementation with this food supplement as part of the daily diet is a useful approach for the management of subjects with slight insulin resistance. If these promising results are confirmed in long-term studies, this food supplement might be recommended to elderly people at a high risk of developing metabolic syndrome and diabetes (i.e., with pre-diabetes, overweight, 45 years or older, have a parent, brother, or sister with type 2 diabetes, are physically active less than 3 times a week) and women of childbearing age with risk factors towards developing gestational diabetes during pregnancy.

Author Contributions: Conceptualization, M.D.; methodology, H.U., C.E., R.P., L.F.D.L. and C.S.; software, H.U., L.F.D.L., C.S. and D.G.B.; validation, C.E., A.D.M. and G.P.; formal analysis, C.E. and R.S.; investigation, H.U., L.F.D.L., C.S. and R.P.; resources, A.B. and A.K.; data curation, A.D.M. and D.G.B.; writing—original draft preparation, H.U., A.K. and G.P.; writing—review and editing, M.D., H.U., A.B., D.G.B. and A.K.; visualization, C.E., R.P., A.D.M., A.B. and R.S.; supervision, M.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was approved by the Scientific Ethics Committee of A.S.L. Napoli 1 CENTRO (Protocol number 222, 12 April 2021) and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000).

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

Data Availability Statement: Not applicable.

Acknowledgments: The authors wish to express their gratitude to the Medical Doctors: Bernardi Giuseppe, Boncompagni Salvatore, Caruso Ciro, Costantino Angelo, Garaffa Elio, Laringe Matteo, Mandaliti Vincenzo, Polistina Claudio, and Santoro Rodolfo (Comegen S.c.S., Società Cooperativa Sociale di Medici di Medicina Generale, Viale Maria Bakunin, 41, 80125 Naples, Italy). Their generous contributions of time and expertise are greatly appreciated. In addition, the authors wish to express their gratitude to Francesca Coppola for her valuable contribution in the collection of the data, and to Eris Scott-Perring for English language support. The authors wish to express their gratitude to Francesca Varvello (Heallo srl, (MI), Italy) for providing the samples tested in this investigation.

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

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Impact of Fecal Microbiota Transplantation on Gut Bacterial Bile Acid Metabolism in Humans

Jessica-Miranda Bustamante [1,†], Tyson Dawson [2,†], Caitlin Loeffler [2], Zara Marfori [1], Julian R. Marchesi [3], Benjamin H. Mullish [3], Christopher C. Thompson [4], Keith A. Crandall [2,5], Ali Rahnavard [2,5], Jessica R. Allegretti [4,*] and Bethany P. Cummings [1,*]

[1] Department of Surgery, School of Medicine, Center for Alimentary and Metabolic Science, University of California, Sacramento, CA 95817, USA
[2] Computational Biology Institute, Department of Biostatistics and Bioinformatics, Milken Institute School of Public Health, The George Washington University, Washington, DC 20052, USA
[3] Division of Digestive Diseases, Department of Metabolism, Digestion and Reproduction, Faculty of Medicine, St. Mary's Hospital Campus, Imperial College London, London W2 1NY, UK
[4] Division of Gastroenterology, Hepatology and Endoscopy, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115, USA
[5] Department of Biostatistics and Bioinformatics, Milken Institute School of Public Health, The George Washington University, Washington, DC 20052, USA
* Correspondence: jallegretti@bwh.harvard.edu (J.R.A.); bpcummings@ucdavis.edu (B.P.C.)
† These authors contributed equally to this work.

Abstract: Fecal microbiota transplantation (FMT) is a promising therapeutic modality for the treatment and prevention of metabolic disease. We previously conducted a double-blind, randomized, placebo-controlled pilot trial of FMT in obese metabolically healthy patients in which we found that FMT enhanced gut bacterial bile acid metabolism and delayed the development of impaired glucose tolerance relative to the placebo control group. Therefore, we conducted a secondary analysis of fecal samples collected from these patients to assess the potential gut microbial species contributing to the effect of FMT to improve metabolic health and increase gut bacterial bile acid metabolism. Fecal samples collected at baseline and after 4 weeks of FMT or placebo treatment underwent shotgun metagenomic analysis. Ultra-high-performance liquid chromatography-mass spectrometry was used to profile fecal bile acids. FMT-enriched bacteria that have been implicated in gut bile acid metabolism included *Desulfovibrio fairfieldensis* and *Clostridium hylemonae*. To identify candidate bacteria involved in gut microbial bile acid metabolism, we assessed correlations between bacterial species abundance and bile acid profile, with a focus on bile acid products of gut bacterial metabolism. *Bacteroides ovatus* and *Phocaeicola dorei* were positively correlated with unconjugated bile acids. *Bifidobacterium adolescentis*, *Collinsella aerofaciens*, and *Faecalibacterium prausnitzii* were positively correlated with secondary bile acids. Together, these data identify several candidate bacteria that may contribute to the metabolic benefits of FMT and gut bacterial bile acid metabolism that requires further functional validation.

Keywords: bile salt hydrolase (BSH); bile acids; gut microbiota; metagenomics; fecal microbiome transplant (FMT)

Citation: Bustamante, J.-M.; Dawson, T.; Loeffler, C.; Marfori, Z.; Marchesi, J.R.; Mullish, B.H.; Thompson, C.C.; Crandall, K.A.; Rahnavard, A.; Allegretti, J.R.; et al. Impact of Fecal Microbiota Transplantation on Gut Bacterial Bile Acid Metabolism in Humans. *Nutrients* **2022**, *14*, 5200. https://doi.org/10.3390/nu14245200

Academic Editor: Abeer M. Mahmoud

Received: 31 October 2022
Accepted: 2 December 2022
Published: 7 December 2022

1. Introduction

Type 2 diabetes mellitus (T2DM) continues to be a worldwide clinical challenge. The gut microbiota plays an important role in determining host metabolic health and has been associated with T2DM. Studies comparing the composition and function of the fecal microbiota from groups who had T2DM, impaired glucose tolerance, or normal glucose tolerance have reported distinct bacterial compositions. For example, *Roseburia* and *Faecalibacterium prausnitzii* are differentially enriched in T2DM [1]. Additionally, decreases in Bacteroidetes and increases in Actinobacteria and Firmicutes are associated with obesity [2,3]. Studies in germ-free mice show that transplantation of the gut microbiota from metabolically

healthy vs. metabolically impaired donors transfers these metabolic phenotypes, pointing to a causative role for the gut microbiota in the pathogenesis of metabolic disease [4,5]. Therefore, the gut microbiome is an attractive target for the treatment and prevention of T2DM.

Fecal microbiota transplantation (FMT) is a potential method to target the gut microbiome for T2DM treatment and prevention [6]. FMT has been shown to successfully treat microbiota-related dysfunction, with the treatment of *Clostridioides difficile* infection being the most notable example of its successful therapeutic use [7,8]. To test the potential utility of FMT for the treatment of metabolic disease, our group previously studied patients with obesity, without metabolic impairment, treated with FMT or placebo. FMT did not induce weight loss but did successfully colonize the gastrointestinal tract of recipients and slowed the development of glucose intolerance compared with placebo, as assessed by mixed meal tolerance testing [9]. The mechanisms for this microbially induced improvement in glucose tolerance are unknown. However, a key mechanism by which the gut microbiota influences host metabolic health is through the production of metabolites, such as short-chain fatty acids (SCFAs) and unconjugated and secondary bile acids. Although SCFA concentrations were not altered by FMT, FMT increased gut bacterial bile acid metabolism compared to placebo resulting in a change in the bile acid profile that mirrored that of the lean donor [10].

Bile acids are a class of bioactive metabolites that signal through bile acid receptors, such as FXR and TGR5, to improve metabolic health. Bile acids are primarily metabolized by the liver and the gut microbiota. Primary bile acids are produced in the liver from cholesterol and are conjugated with taurine or glycine prior to secretion into the gut lumen. Primary bile acids are converted into secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), by gut bacteria. Bile acids vary in their affinity for bile acid receptors. Therefore, alterations in the bile acid profile can influence metabolic health by altering bile acid receptor signaling. In particular, DCA and LCA are the strongest ligands for TGR5 [11–14]. Indeed, studies investigating the impact of gut microbiota on metabolic disease often identify gut bacterial bile acid production as a key mechanistic mediator. For example, dietary fiber supplementation has been reported to enhance gut bacterial 6-α-hydroxylation to improve metabolic phenotypes in mice [15]. Furthermore, recent work reports that enhancing gut bacterial bile acid deconjugation through the use of genetically modified microbes improves metabolic parameters in mice [16].

A key pathway in gut bacterial bile acid metabolism is the conversion of conjugated primary bile acids to secondary bile acids through deconjugation followed by 7-α-dehydroxylation. Primary bile acids are first deconjugated by the enzyme bile salt hydrolase (BSH) [17,18]. BSH expression has been identified across all major bacterial divisions and archaeal species in the gut, and elevations in BSH activity improve metabolic outcomes [19]. Furthermore, BSH activity enhances bacterial survival [19]. Therefore, BSH may also be a key determinant of the efficacy by which probiotics and FMT are able to successfully colonize the host [20]. However, the regulation of BSH expression is poorly understood. Unconjugated bile acids are converted to secondary bile acids through 7-α-dehydroxylation, which is a multi-step process that is less widely dispersed throughout the gut microbiome relative to BSH [17,18]. Nevertheless, the gut bacterial species and genes responsible for 7-α-dehydroxylation are still incompletely defined.

Research suggests FMT increases gut microbial diversity and the abundance of beneficial bacteria. Indeed, we found that patients who received FMT show sustained shifts in gut microbiota profiles toward those of the donor, as determined by 16S rRNA gene sequencing. Additionally, bile acid profiles resembled that of the donor [10]. Importantly, this shift in bile acid profile also coincided with a slowing of glycemic impairment compared with placebo [9]. The results from this clinical pilot, which evaluated the effectiveness of FMT in obese metabolically healthy patients, provide an ideal study set to identify gut bacteria involved in gut bacterial bile acid metabolism. Therefore, we assessed the impact of FMT on gut bacterial composition by metagenomics to better understand the dynamic alterations induced by FMT. Furthermore, we assessed the correlation between bacterial abundance

determined by metagenomics with bile acid levels, assessed in the same samples, to identify putative bacterial species that may contribute to gut microbial bile acid metabolism.

2. Materials and Methods

2.1. Sample Collection

Secondary analysis was conducted on a single-center, double-blind, randomized, placebo-controlled pilot trial of FMT in obese metabolically normal/healthy patients (body mass index (BMI), 35 kg/m^2 or higher without diabetes, metabolic syndrome, or non-alcoholic fatty liver disease). Briefly, patients were randomized 1:1 to receive FMT (via an induction dose of 30 FMT capsules followed by two maintenance doses of 12 capsules at week 4 and week 8) or an identical placebo capsule. A single healthy lean (BMI 17.5 kg/m^2) donor was used to generate FMT capsules. A total of 22 patients were enrolled, 11 in each arm, and primarily female [10]. Samples collected at baseline (prior to FMT intervention) and after 4 weeks of intervention were available from 8 placebo and 11 FMT patients. Two patients in the placebo group withdrew from the study, and one placebo sample was unavailable at the 4-week time point. We focused on the 4-week time point because this was the time point at which the most substantial FMT-induced change in the bile acid profile was detected [10].

2.2. Microbiota Analysis

Fecal samples were shipped frozen to the George Washington University Genomics Core for processing. Each sample underwent DNA and RNA extraction in parallel from 250 mg of fecal material using a ZymoBIOMICS DNA/RNA Miniprep Kit (Zymo Research Corporation, Irvine, CA, USA). The resulting DNA was quality controlled using a Thermo Fisher Qubit 3.0 High Sensitivity DNA kit (Life Technologies, Carlsbad, CA, USA) and standardized to 0.2 ng/µL for library construction. Sequencing libraries were prepared, along with a ZymoBIOMICS Microbial Community DNA standard, using a Nextera XT DNA Library Prep kit (Illumina, San Diego, CA, USA) following Illumina's recommended guidelines. Libraries were quality controlled using a Thermo Fisher Qubit 3.0 High Sensitivity DNA kit and an Agilent 2100 Bioanalyzer High Sensitivity DNA kit (Agilent, Santa Clara, CA, USA) and were subsequently sequenced as paired-end, 2 × 150 bp, using a NextSeq 500 Mid-Output kit (Illumina, San Diego, CA, USA), with a 1% phi X sequencing control spike-in.

2.3. Data Analysis and Statistics

Metagenomic shotgun sequencing read quality was assessed using FastQC and MultiQC [21,22], and host reads were filtered using kneaddata with low quality (Phred scores < 28) ends and reads trimmed for downstream analyses. Functional pathways of associated microbes were determined using omePath [23]. Functional associations between metabolites, clinical phenotypes, and microbes were assessed using Tweedievers [24]. Data are presented as the mean ± SEM, and statistical analyses were performed using GraphPad Prism 9.4.1. Data were analyzed by non-parametric Wilcoxon matched-pairs signed rank test. Multiple corrections of statistical tests were applied using the Benjamini and Hochberg false discovery rate (FDR), and differences were considered significant at $p \leq 0.05$ unless otherwise noted.

3. Results

3.1. Microbial Diversity

Patient fecal samples obtained at baseline (prior to FMT intervention) and after 4 weeks of intervention were assessed by shotgun metagenomics. The metagenomic sequencing resulted in an average of 4,015,023 reads per sample, with a minimum of 2,280,276 reads and a maximum of 5,818,393 reads. Of these, 0.031235% were, on average, the host reads, leaving a minimum of 2,279,728 quality microbial reads for microbiome characterization with an average of 4,013,907 quality microbial reads per host individual.

3.2. Impact of FMT on Gut Microbial Composition at 4 Weeks after the Initiation of Intervention

There were no significant baseline clinical differences between FMT and placebo groups [10]. We focused on the fecal sample collected at baseline vs. 4 weeks after the initiation of FMT as this was the time point at which the changes in bile acid levels were most significant [10]. No significant differences in the relative abundance of bacteria were noted at the phyla level (Figures 1 and 2A). At the genus level, FMT-enriched *Paraprevotella* and *Longibaculum* (Figure 2B,C, $p < 0.05$) compared to placebo. On the species level, FMT tended to increase the relative abundance of *Clostridium hylemonae*, a bacterial species known to convert primary to secondary bile acids (Figure 2D) [17,18]. Finally, FMT increased *Desulfovibrio fairfieldensis* compared with placebo (Figure 2E, $p < 0.05$). Of note, *Paraprevotella*, *Longibaculum*, *Clostridium hylemonae*, and *Desulfovibrio fairfieldensis* did not differ between groups at the baseline.

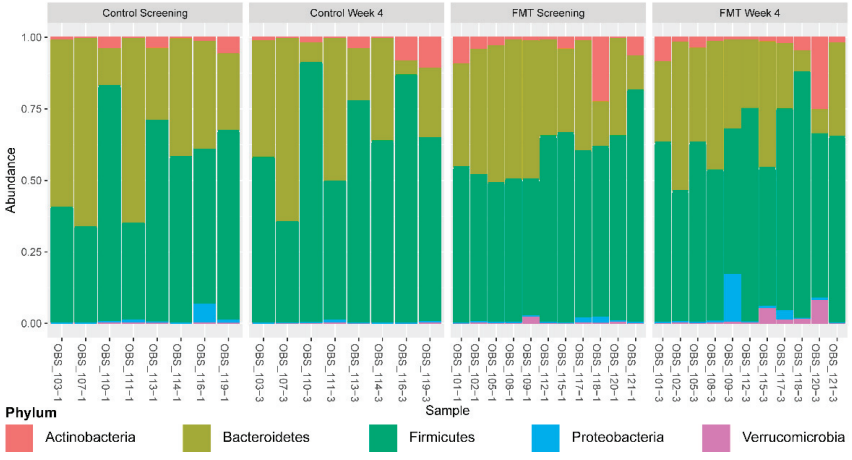

Figure 1. Phylum-level relative abundances across all individual patients. Patient identifiers ending in 1 represent baseline and ending in 3 represent the 4-week time point. Plots are faceted by treatment and time point.

3.3. Impact of FMT on Gene Enrichment and Correlations with Secondary Bile Acid Production

As some of the bacterial species enriched by FMT are implicated in bile acid metabolism, we next determined the effect of FMT on bacterial bile acid metabolic gene copy number. Starting with a broad overview of the impact of FMT on gut microbial gene abundance, we performed pathway analysis using *omePath* of the metagenomic data (Figure 3A). FMT-enriched genes involved in cell proteolysis pathways. Taking a closer look at bile acid metabolism, FMT did not impact gene abundance for most known gut bacterial bile acid metabolic genes, except for a reduction in *BaiB* and *BaiE*. These changes were noted at 4 weeks and not at baseline in the FMT group compared to the placebo. FMT did not impact the gene abundance of other genes involved in bile acid 7-α-dehydroxylation, including *BaiCD*, *BaiA2*, *BaiF*, and *BaiH*. Further work using metatranscriptomics is warranted to determine the impact of FMT on bacterial bile acid metabolic gene expression.

To identify candidate bacteria involved in gut bacterial bile acid metabolism, we assessed correlations between bacterial species abundance and bile acid profile, with a focus on bile acid products of gut bacterial metabolism, namely unconjugated bile acids, and the secondary bile acids, DCA and LCA. Bile acid levels were measured in the same samples used for metagenomics analysis, as previously described [10]. The impact of FMT on bile acid levels in this sample set has been previously reported [10]. We focused on bacterial species that were positively correlated with bile acid sub-types that are produced, at least in part, through interactions with the gut microbiota with a *p*-value less than or equal to 0.08.

Bacterial species that met these criteria are presented in Figure 3B. *Phocaeicola dorei* and *Bacteroides ovatus* were positively correlated with unconjugated chenodeoxycholic acid (CDCA) ($p = 7.47 \times 10^{-45}$ and 2.50×10^{-8}). *Bifidobacterium adolescentis* and *Collinsella aerofaciens* were positively correlated with the production of DCA (specifically, the glycine-conjugated form, GDCA) ($p = 0.0123$ and 0.0634). The same strain of *B. ovatus* was positively correlated with unconjugated cholic acid (CA) ($p = 0.0317$). Lastly, *Faecalibacterium prausnitzii* was positively correlated with LCA ($p = 0.0634$). These data point to a potential role for *Phocaeicola dorei* and *B. ovatus* in bile acid deconjugation. Further, these data suggest that *Bif. adolescentis*, *C. aerofaciens*, and *F. prausnitzii* may play a role in the conversion of primary to secondary bile acids, which requires further functional validation.

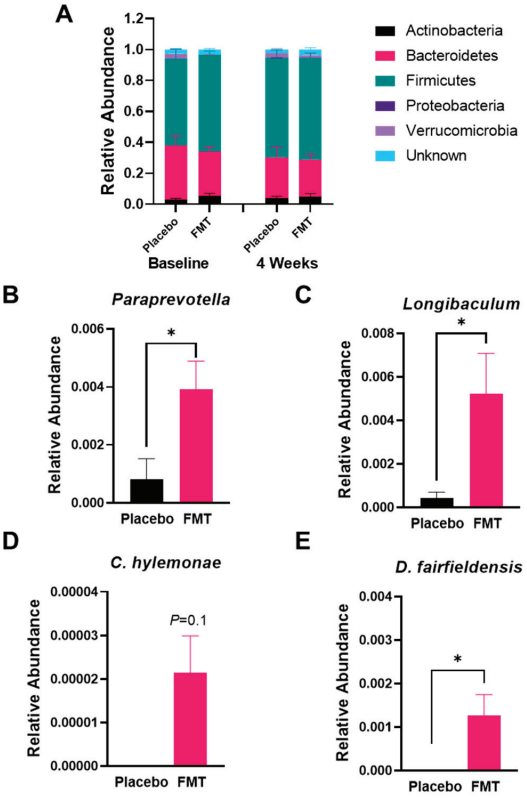

Figure 2. Impact of FMT on gut microbial composition. (**A**) Relative abundance of each bacterial phylum. Relative abundance of *Paraprevotella* (**B**) and *Longibaculum* (**C**), *Clostridium hylemonae* (**D**), and *Desulfovibrio fairfieldensis* (**E**) in fecal samples from placebo vs. FMT at baseline and after 4 weeks of treatment. Data presented as mean ± SEM. * $p < 0.05$.

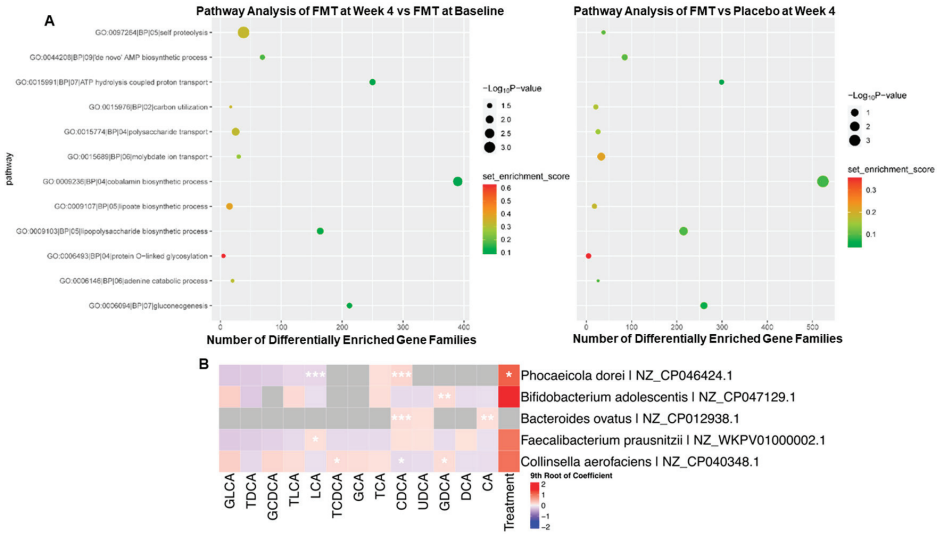

Figure 3. Association between bile acids and bacterial species. (**A**) Assessment of pathways enriched by FMT relative to baseline (**left**) and relative to placebo control after 4 weeks of treatment (**right**). (**B**) Bacterial species and strains that are correlated with gut bacterial-derived bile acids. * $p < 0.2$, ** $p < 0.05$, *** $p < 0.001$.

4. Discussion

In the present study, we utilized a fecal sample set from patients receiving FMT or placebo that exhibited alterations in gut bacterial bile acid metabolism to improve our understanding of the gut bacterial species involved in gut bacterial bile acid metabolism and how these pathways are dynamically regulated by FMT. Using metagenomics, we identified an enrichment of *Paraprevotella*, *Longibaculum*, *Desulfovibrio fairfieldensis*, and *Clostridium hylemonae* in response to FMT. Furthermore, through the assessment of correlations between fecal bile acid levels and bacterial species relative abundances, we identified *Bifidobacterium adolescentis*, *Bacteroides ovatus*, *Faecalibacterium prausnitzi*, and *Phocaeicola dorei* as potentially contributing to gut bacterial bile acid metabolism. Further work is needed to better understand secondary bile acid metabolism, its roles in metabolic disease, and how it can be manipulated through FMT.

The effect of FMT to enrich *Paraprevotella, Longibaculum*, *C. hylemonae*, and *D. fairfieldensis* may have contributed to the effect of FMT to enhance gut microbial bile acid metabolism and/or slow the development of glucose intolerance. For example, members of the genera *Clostridium* are the predominant human intestinal species thought to perform 7-α-dehydroxylation of primary bile acids [25]. Additionally, *C. hylemonae* has been shown to convert CA into DCA in vitro [26]. Furthermore, *Paraprevotella* abundance was significantly increased after FMT in individuals with functional constipation whose changes in fecal microbiome compositions were measured before and after FMT. This increase in *Paraprevotella* abundance correlated with improved relief of clinical symptoms measured by three different clinical scales for constipation, suggesting *Paraprevotella* could improve metabolic dysregulation through gastric motility [27]. The role of *Longibaculum* in host metabolic health is poorly defined; however, dietary fiber supplementation has been shown to enrich for *Longibaculum* [28]. *D. fairfieldensis* is a Gram-negative anaerobic bacillus that has been implicated in bile acid metabolism. Interestingly, *D. fairfieldensis* bacteremia was found to be associated with choledocholithiasis in a case report [29]. Furthermore, a recent study reports that *Desulfovibrionales* are enriched in patients with cholelithiasis. Further, the administration of *Desulfovibrionales* to mice with antibiotic-induced depletion of the gut microbiome increased secondary bile acid production [30]. *Desulfovibrionales* can reduce

taurine into H_2S, which has been suggested to facilitate 7-α-dehydroxylation [31]. Together, these data suggest that *Desulfovibrionales*, and in particular *D. fairfieldensis*, may play a role in gut bile acid metabolism. Further, these data highlight the potentially important cooperative interactions among bacteria that facilitate gut microbial bile acid metabolism.

In this study, we identified five bacteria that were positively correlated with gut microbiome-derived bile acids. Specifically, *Bifidobacterium adolescentis* and *Collinsella aerofaciens* were positively correlated with DCA. *Bacteroides ovatus* was positively correlated with unconjugated CDCA and CA. *Phocaeicola dorei* was positively correlated with unconjugated CDCA, and *Faecalibacterium prausnitzii* was positively correlated with LCA. Thus, our data suggest that *Phocaeicola dorei* and *Bacteroides ovatus* may perform bile acid deconjugation. Consistent with this, previous work reports that several *Bacteroides* strains, including strains of *B. ovatus*, express BSH [32]. Whether *Phocaeicola dorei* can perform bile acid deconjugation is unknown and requires further testing. Interestingly, a previous study identified a correlation between *Phocaeicola dorei*, also named *Bacteroides dorei*, and the risk of developing type 1 diabetes [33], suggesting a potential metabolic role for this species. The bacteria that were positively correlated with secondary bile acids (*Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*) are known to have BSH functions [34,35]. *Faecalibacterium prausnitzii* has been connected to anti-inflammatory effects and improvement of intestinal barrier function [36,37]. A role for *Collinsella aerofaciens* in the production of DCA has not been previously tested. However, *Collinsella aerofaciens*, previously known as *Eubacterium aerofaciens*, was found to have NADP-dependent 7-β-hydroxysteriod dehydrogenase activity, which is necessary for the production of hydrophilic secondary bile acids such as ursodeoxycholic acid [38].

The advantages of this study include the application of metagenomics to the analysis of the gut microbiome of individuals receiving FMT or placebo control. Additionally, the results from this secondary analysis are from individuals with obesity such that bacteria identified from this specific population can better inform FMT for the treatment of obesity and metabolic disease. Limitations of this study include the small sample size. While bile acid gene abundance was studied, metatranscriptomics analysis is needed to assess the impact of FMT on gene expression. Further work is needed to functionally validate bacteria identified as potentially contributing to the effects of FMT on gut bacterial bile acid metabolism. Together, these data demonstrate that FMT can alter the composition of bile acids and bacterial communities in the gut microbiome.

Author Contributions: Investigation: J.-M.B., T.D., C.L., Z.M., J.R.M., K.A.C., A.R., B.H.M., J.R.A. and B.P.C. Conceptualization, Resources: J.R.A. and B.P.C. Methodology: J.-M.B., T.D., C.L., Z.M., J.R.M., K.A.C., A.R., B.H.M., J.R.A. and B.P.C. Formal Analysis: J.-M.B., T.D., C.L., Z.M., J.R.M., K.A.C., A.R. and B.P.C. Writing—Original Draft Preparation: J.-M.B. Writing—Review and Editing: J.-M.B., T.D., C.L., Z.M., J.R.M., C.C.T., K.A.C., A.R., B.H.M., J.R.A. and B.P.C. Supervision, Funding Acquisition: K.A.C., A.R. and B.P.C. All authors have read and agreed to the published version of the manuscript.

Funding: Research reported in this publication was supported by the National Center for Complementary and Integrative Health of the National Institutes of Health under Award number R21AT010956 to B.P.C. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Metabolomics studies were performed at the MRC-NIHR National Phenome Centre at Imperial College London; this center receives financial support from the Medical Research Council (MRC) and National Institute of Health Research (NIHR) (grant number MC_PC_12025). B.H.M. is the recipient of an NIHR Academic Clinical Lectureship (CL-2019-21-002) and was formerly in receipt of an MRC Clinical Research Training Fellowship (MR/R000875/1). The Division of Digestive Diseases and MRC-NIHR National Phenome Centre at Imperial College London receive financial and infrastructure support from the NIHR Imperial Biomedical Research Centre (BRC) based at Imperial College Healthcare NHS Trust and Imperial College London.

Institutional Review Board Statement: The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board at the Brigham and Women's Hospital, and all patients provided written informed consent before participation (NCT02741518). In addition, Food and Drug Administration approval via an investigational new drug application (16936, 2016) was obtained. All authors had access to the study data and approved the final manuscript.

Informed Consent Statement: All patients provided written informed consent before participation.

Data Availability Statement: Sequence data generated by this project are deposited in NCBI Sequence Read Archive (SRA) and associated with BioProject PRJNA904790.

Acknowledgments: We thank Castle Raley and the George Washington Genomics Core for sample processing and sequencing for metagenomics.

Conflicts of Interest: J.-M.B., C.L., Z.M., J.R.M., K.A.C., A.R. and B.P.C. have no relevant conflict of interest to declare. J.R.A. consults for and has research support from Finch Therapeutics Group, Janssen, Pfizer, Abbvie, Iterative Sopes, Seres Therapeutics, Ferring, Merck, Bristol Myer Squibb and has research support from Pfizer and Merck. T.D. has research support from AMPEL BioSolutions. B.H.M. has received consultancy fees from Finch Therapeutics Group and Ferring Pharmaceuticals. C.C.T. consults for Apollo Endosurgery, Boston Scientific, Medtronic, Enterasense Ltd., EnVision Endoscopy, Fractyl, Fujifilm, GI Dynamics, GI Windows, Lumendi, Olympus, USGI Medical, Xenter, Endoquest Robotics. He has received Research Support from Apollo Endosurgery, Boston Scientific, ERBE, Fujifilm, GI Dynamics, Lumedi Olympus, USGI Medical. He serves on Advisory Boards for Fractyl, Fujifilm, USGI Medical, Xenter and Endoquest Robotics. He is a founder, board member and receives ownership interest from Enterasense Ltd., EnVision Endoscopy and GI Windows, He is on a speakers bureau for Boston Scientific, Fujifilm and Olympus. He receives royalty payments from GI Windows, EndoSim and Enterasense Ltd.

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Dahl Salt-Resistant Rat Is Protected against Hypertension during Diet-Induced Obesity

Soyung Lee [1,2,3,†], Sungmin Jang [1,2,3,†], Jee Young Kim [1,2,3] and Inkyeom Kim [1,2,3,*]

[1] Department of Pharmacology, School of Medicine, Kyungpook National University, Daegu 41944, Korea
[2] Cardiovascular Research Institute, Kyungpook National University, Daegu 41944, Korea
[3] BK21 Plus KNU Biomedical Convergence Program, Department of Biomedical Science, Kyungpook National University, Daegu 41944, Korea
* Correspondence: inkim@knu.ac.kr; Tel.: +82-10-6546-6933
† These authors contributed equally to this work.

Abstract: A high-fat diet (HFD) frequently causes obesity-induced hypertension. Because Dahl salt-resistant rats are protected against hypertension after high-salt or high-fructose intake, it is of interest whether this model also protects against hypertension after diet-induced obesity. We tested the hypothesis that Dahl salt-resistant rat protects against hypertension during diet-induced obesity. Dahl salt-sensitive (SS) and Dahl salt-resistant (SR) rats were fed a HFD (60% fat) or a chow diet (CD; 8% fat) for 12 weeks. We measured blood pressure using the tail-cuff method. The paraffin sections of thoracic perivascular adipose tissue (tPVAT) were stained with hematoxylin/eosin and trichrome. The expression of genes in the tPVAT and kidneys were measured by reverse transcription-quantitative polymerase chain reaction. The HFD induced hypertension in SS ($p < 0.01$) but not SR rats, although it increased body weight gain ($p < 0.05$) and tPVAT weight ($p < 0.01$) in both rats. The HFD did not affect the expression of genes related to any of the adipocyte markers in both rats, although SR rats had reduced beige adipocyte marker Tmem26 levels ($p < 0.01$) and increased anti-inflammatory cytokine adiponectin ($p < 0.05$) as compared with SS rat. The HFD did not affect the mRNA expression of contractile factors in the tPVAT of SS and SR rats. SR rats are protected against hypertension during diet-induced obesity. This result implies that the genetic trait determining salt sensitivity may also determine fructose and fat sensitivity and that it is associated with the prevention of hypertension.

Keywords: Dahl salt-resistant rat; Dahl salt-sensitive rat; hypertension; obesity; perivascular adipose tissue

Citation: Lee, S.; Jang, S.; Kim, J.Y.; Kim, I. Dahl Salt-Resistant Rat Is Protected against Hypertension during Diet-Induced Obesity. *Nutrients* **2022**, *14*, 3843. https://doi.org/10.3390/nu14183843

Academic Editors: Shane Phillips and Abeer M. Mahmoud

Received: 12 August 2022
Accepted: 14 September 2022
Published: 16 September 2022

1. Introduction

In many countries, the prevalence of obesity is increasing, and one-third of the world-wide population is described as obese or overweight [1]. Obesity is currently considered a pandemic and is frequently associated with metabolic syndromes such as hypertension, insulin resistance, and abnormal blood lipid levels [2,3]. A high-fat diet (HFD) increases excessive body fat storage and is a major factor in obesity [4]. This diet represents a risk factor for the development and/or worsening of several chronic diseases, including cardiovascular diseases [5].

Perivascular adipose tissue (PVAT) is the adipose tissue that surrounds the blood vessels. PVAT differs based on the species and anatomic location and is generally classified as brown (thermoactive adipocytes), beige (brown-like adipocytes), or white adipose tissue (lipid-storing adipocytes) [6]. PVAT releases a wide range of adipokines, vasoactive and pro-/anti-inflammatory mediators that influence vascular function in a paracrine manner [7]. PVAT plays a beneficial role by releasing anticontractile factors such as hydrogen sulfide, adiponectin, and nitric oxide (NO) in equilibrium. A great number of contractile factors are produced in obesity, such as angiotensin II, chemerin, serotonin, dopamine, norepinephrine, calpastatin, and so on [8]. Moreover, obesity is frequently associated with structural

and functional alterations in PVAT, leading to vascular dysfunction that involves the endothelium and smooth muscle as well as increased overall cardiovascular risk [9].

Hypertensive patients account for 25% of all patients with chronic kidney disease (CKD), and hypertensive patients with obesity are at the greatest risk for developing CKD [10,11]. The development of obesity leads to significant lipid deposits around or within non-adipose tissues and organs (ectopic fat), which impairs both tissue and organ function [12]. Obesity also affects the kidney, which has assigned roles in dyslipidemia, the production of adipokines and angiotensin II, oxidative stress, hyperfiltration, immune activation, and lipotoxicity [13,14]. The activation of the renin-angiotensin-aldosterone system (RAAS) is the main mechanism by which obesity induces the development of high blood pressure [15]. Because experimental results from animals and humans have suggested the activation of the RAAS in hypertension with obesity, the RAAS is considered important in controlling blood pressure in obesity [16].

A recent study showed that a HFD increases blood pressure in the Dahl salt-sensitive (SS) rat model [17]. The SS rat has been regarded as the most popular model of human salt-sensitive hypertension. Salt-sensitive hypertension is more likely to cause multiple organ damage and results in a higher prevalence of cardiovascular and renal diseases among hypertensive subjects [18]. Several studies have shown that the Dahl salt-resistant (SR) rat remains normotensive in resistance to diet-induced hypertension, such as with high salt or high fructose intake, as compared with the SS rat [19]. However, it is of interest whether the SR rat is protected against hypertension after diet-induced obesity. Therefore, we tested the hypothesis that SR rat is protected against hypertension during diet-induced obesity.

2. Materials and Methods

2.1. Animals

The Institutional Review Board of Kyungpook National University approved the care and use of animals (approval No. 2021-0192). Every effort was made to minimize both the number of animals used and their suffering. Seven-week-old male SS rats (DIS/EisSlc; Dahl-Iwai S) and SR rats (DIR/EisSlc; Dahl-Iwai R) were purchased from Japan SLC, Inc. (Hamamatsu, Shizoka, Japan, n = 6). The rats were acclimatized for a couple of weeks before being randomly assigned into one of two groups fed with a chow diet (CD; containing 8.6% fat; SAFE, Paris, France) [20] or a HFD (60% fat, Research Diets, Inc., New Brunswick, NJ, USA) and allowed free access to food and tap water for 12 weeks. The body weight of the rats was monitored weekly for 12 weeks using an electronic scale (KB-5000, A&D KOREA Ltd., Seoul, Korea). Caloric intake was calculated by multiplying food intake (g/day) by 3.1 kcal/g for the CD groups and 5.2 kcal/g for the HFD groups, respectively. The rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) for sacrifice, after which, tissues were obtained, frozen in liquid nitrogen, and stored at −80 °C until further study.

2.2. Blood Pressure Measurements

We measured the systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the rats using the tail-cuff method. Rats were preheated on a hotplate at 35 °C for 10 min and then placed in plastic restrainers. We attached a cuff with a pneumatic pulse sensor around the tail. The CODA system (Kent Scientific Corporation, Torrington, CT, USA) was used to record blood pressure values with heating. Blood pressure measurements were averaged from at least ten consecutive readings obtained from each rat [20].

2.3. Histological Analysis

After the thoracic aorta including PVAT had carefully been excised, the PVAT was isolated from the thoracic aorta and weighed. For staining hematoxylin and eosin (H&E) and trichrome, thoracic aorta and the surrounding thoracic PVAT (tPVAT) were fixed in 4% formalin overnight and then dehydrated and embedded in paraffin using conventional methods. Paraffin-embedded samples were sectioned to a thickness of 2.0 μm. After

staining, slides were examined with light microscopy. We used ImageJ software (National Institutes of Health, Bethesda, MD, USA) to measure the area of the adipocytes. The calculated areas were multiplied by a conversion factor to determine the cross-sectional area of the adipocytes in μm^2.

2.4. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Tissues (around 100 mg) were homogenized in liquid nitrogen with a glass homogenizer. Total RNA was extracted using QIAzol® Lysis Reagent (QIAGEN Science, Germantown, MD, USA) according to the manufacturer's instructions. RNA was converted into cDNA using the RevertAid™ first-strand cDNA synthesis kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Next, we conducted a quantitative polymerase chain reaction using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The reaction solution (20 µL) contained 10 µL of SYBR Green master mix (New England Biolabs, Ipswich, MA, USA), 4 µL of cDNA, and 6 µL of primer set (200 nmol/L). The PCR reactions were conducted as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles at 95 °C for 15 s, followed by 1 min at 60 °C. The relative expression levels were determined as the Δcycle threshold (ΔCt) [19]. All primer sets used in the present study are shown in Supplemental Table S1.

2.5. Measurement of Cytokine Levels

We used enzyme-linked immunosorbent assay (ELISA) kits to analyze the following in PVAT: interleukin-6 (IL-6; BMS625; ThermoFisher Scientific), tumor necrosis factor α (TNFα; ab100785; abcam, Cambridge, UK), and adiponectin (RRP300; R&D Systems, Inc., Minneapolis, MN, USA). The optical density was read at 450 nm. The concentrations of IL-6, TNFα, and adiponectin were calculated in accordance with the standard curves.

2.6. Statistics

Data were expressed as mean ± standard error of mean (SEM). Statistical analyses were performed using Graph Pad Prism 7 (GraphPad Software, San Diego, CA, USA) with a p value of <0.05 considered significant.

3. Results

3.1. HFD Increased Blood Pressure Only in SS Rats Regardless of Accelerated Body Weight Gain in Both Rats

To determine whether an HFD had an effect on blood pressure and body weight, we measured the SBP, DBP, body weight, water intake, food intake, and calorie intake in the rats during the HFD for 12 weeks. The HFD significantly increased the blood pressure in SS rats but not SR rats (Figure 1A). Compared with the CD groups, the HFD steadily increased body weight (Figure 1B). The HFD did not affect water intake (Figure 1C). Although the HFD groups ate less than the CD groups did (Figure 1D), there was no difference in calorie intake between the CD and HFD groups (Figure 1E).

3.2. HFD Increased the Adipocyte Area in the SS Rats Regardless of Increased tPVAT Weight in Both Rats

We measured the tPVAT weight and adipocyte area to evaluate the effect of the HFD on tPVAT characteristics. The HFD significantly increased fat deposits and tPVAT weight in both SS and SR rats (Figure 2A,B). H&E staining of tPVAT revealed that the HFD increased the adipocyte area in SS but not SR rats, whereas trichrome staining revealed that the HFD did not affect fibrosis (Figure 2C). Quantification of the adipocyte area supported this observation (Figure 2D).

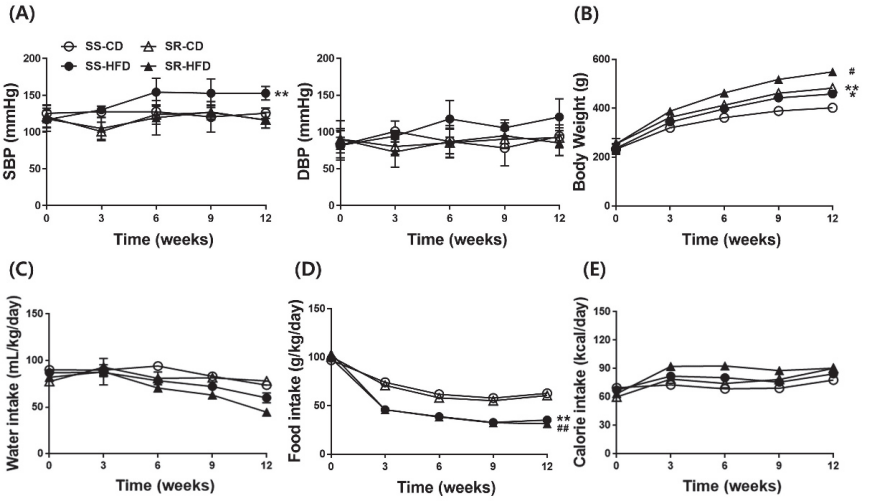

Figure 1. The effects of a high-fat diet on systolic blood pressure (SBP), diastolic blood pressure (DBP), body weight, water intake, food intake, and calorie intake in Dahl salt-sensitive (SS) and salt-resistant (SR) rats. SS and SR rats were fed either a chow diet (CD, 8.6% fat) or a high-fat diet (HFD, 60% fat) for 12 weeks. (**A**) The HFD increased the SBP in SS rats but not in SR rats. (**B**) The HFD increased body weight gain in both the SS and SR rats. (**C**–**E**) Water intake, food intake, and calorie intake are shown. As food intake decreased in the HFD groups, there was no difference in calorie intake between the CD and HFD groups. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's *post hoc* multiple comparisons test. * $p < 0.05$ and ** $p < 0.01$ vs. the SS CD group. # $p < 0.05$ and ## $p < 0.01$ vs. the SR CD group. ○, SS-CD; ●, SS-HFD; △, SR-CD; ▲, SR-HFD.

3.3. HFD Did Not Affect the Expression of Genes Related to Adipocyte Markers in tPVAT of SS and SR Rats

Based on the results that the HFD increased the adipocyte area only in the SS rats, we measured the expression of genes related to adipocyte markers, *Ucp1*, *Pgc1α*, and *Pparγ*, representing brown fat; *Tmem26* representing beige fat; and *Leptin* representing white fat in tPVAT from the SS and SR rats. SS rats had a higher gene expression of *Tmem26* as compared with SR rats fed a CD (Figure 3D), with no significant change in *Ucp1*, *Pgc1α*, *Pparγ*, or *Leptin* (Figure 3).

3.4. HFD Did Not Affect the Expression of the Inflammatory or Contractile Factors in tPVAT of SS and SR Rats

To determine whether the HFD affected the inflammatory factors (IL-6, TNFα, and adiponectin) in tPVAT, we conducted ELISA analysis (Figure 4). The HFD did not affect the proinflammatory factors (IL-6 and TNFα) in tPVAT of SS and SR rats (Figure 4A,B). The tissue level of the anti-inflammatory factor (adiponectin) in tPVAT was higher in SR rats than in SS rats fed a CD (Figure 4C). We measured the expression of genes related to contractile factors (angiotensin II, norepinephrine, chemerin, and serotonin) to investigate whether the HFD affected the contractile factors in tPVAT (Figure 5). The HFD did not affect the gene expressions of *Ace*, *Angiotensinogen*, *Tyrosine hydroxylase*, *Rarres2*, *Cmklr1*, or *Slc6a4* in tPVAT of SS or SR rats.

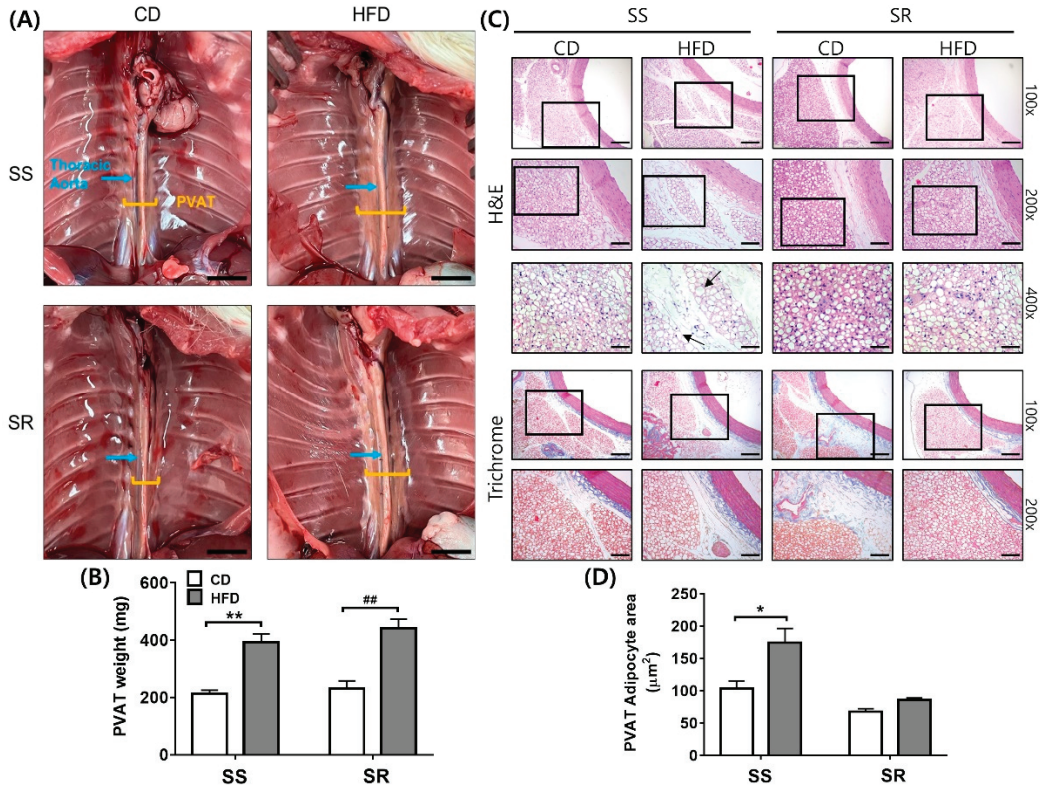

Figure 2. The effects of a high-fat diet on fat deposits, weight, histological characteristics, and adipocyte area of the thoracic perivascular adipose tissues (tPVAT) in SS and SR rats. (**A**) Representative pictures visualizing tPVAT from the SS and SR rats fed a CD or HFD for 12 weeks. The HFD increased fat deposits (**A**) and PVAT weight (**B**) in both the SS and SR rats. (**C**,**D**) Representative microscopic images of the thoracic aortas and tPVAT from the SS and SR rats fed a CD or HFD for 12 weeks. The thoracic aortas and tPVAT sections were stained with hematoxylin and eosin (H&E, upper) and trichrome (lower) stains. The scale bars for 100×, 200×, and 400× magnification were 200, 100, and 50 μm, respectively. The HFD increased the PVAT adipocyte area (black arrow) in SS rats but not SR rats. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's post hoc multiple comparisons test. * $p < 0.05$ and ** $p < 0.01$ vs. the SS CD group. ## $p < 0.01$ vs. the SR CD group.

3.5. HFD Did Not Affect the Expression of Genes Related to RAAS in the Kidney of SS and SR Rats

Based on our findings that the HFD increased blood pressure only in SS rats without affecting the expression of contractile factors in tPVAT, we investigated whether the HFD increased the mRNA expression of RAAS genes in the kidney. The HFD did not increase the mRNA expression of *Renin*, *Ace*, *Ace2*, or *Angiotensinogen* (Figure 6), nor that of angiotensin receptors (*At1ar*, *At1br*, *At2r*, *Mas1*; Figure 7). We also investigated the effect of HFD on the mRNA expression of NADPH oxidase-related factors in the kidneys of SS and SR rats. These data showed that there were no differences in gene expression between the CD and HFD groups (Figure S1).

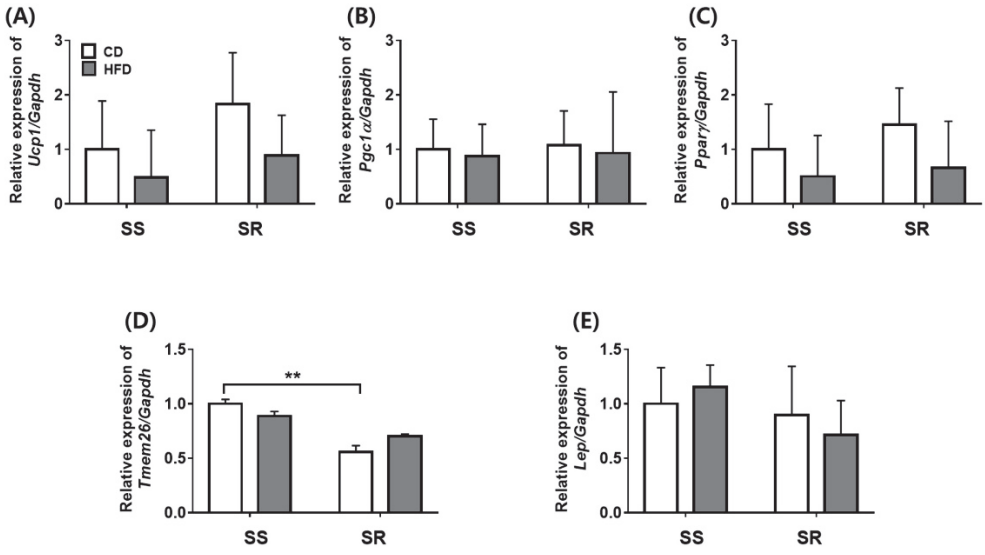

Figure 3. The effects of a high-fat diet on the expression of adipocyte marker genes in PVAT from SS and SR rats. The mRNA expression of brown adipocyte-related genes such as *uncoupling protein 1* (*Ucp1*, **A**), *peroxisome proliferator-activated receptor γ coactivator 1-α* (*Pgc-1α*, **B**), and *peroxisome proliferator-activated receptor γ* (*Pparγ*, **C**), a beige adipocyte-related gene such as *transmembrane protein 26* (*Tmem26*, **D**), and a white adipocyte-related gene such as *Leptin* (**E**) were measured by RT-qPCR in PVAT from SS and SR rats fed a CD or HFD for 12 weeks. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's post hoc multiple comparisons test. ** $p < 0.01$ vs. the SS CD group.

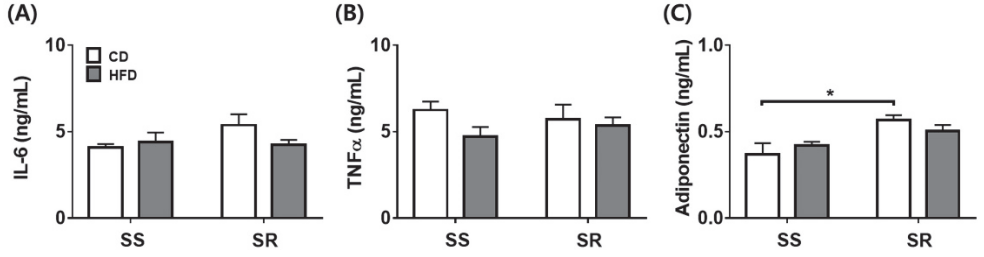

Figure 4. The effects of a high-fat diet on adipose tissue derived inflammatory factors in PVAT from SS and SR rats. Tissue levels of proinflammatory cytokines (interleukin-6 [IL-6, **A**] and tumor necrosis factor α [TNFα, **B**]) and an anti-inflammatory cytokine (adiponectin, **C**) were detected via enzyme-linked immunosorbent assay (ELISA) in SS and SR rats fed a CD or HFD for 12 weeks. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's post hoc multiple comparisons test. * $p < 0.05$ vs. the SS CD group.

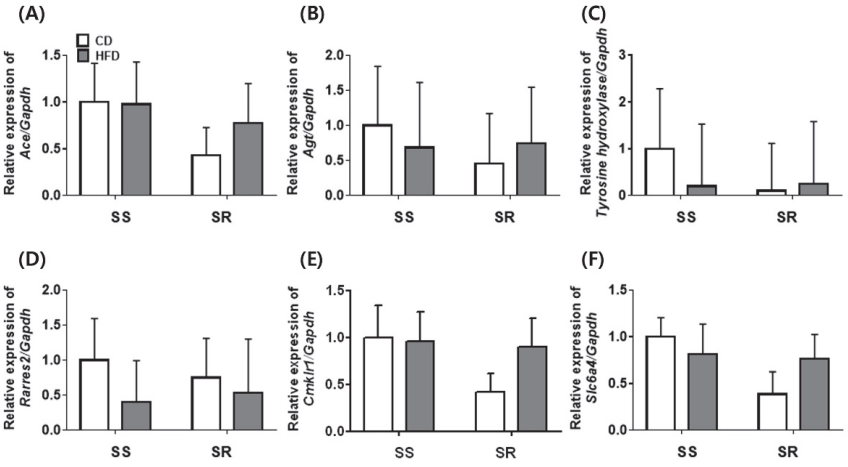

Figure 5. The effects of a high-fat diet on the expression of genes related to contractile factors in PVAT from SS and SR rats. The mRNA expression of angiotensin II-related genes such as *angiotensin-converting enzyme* (*Ace*, **A**) and *angiotensinogen* (*Agt*, **B**), a norepinephrine-synthesizing gene such as *tyrosine hydroxylase* (**C**), chemerin-related genes such as *rarres2* (**D**) and chemerin receptor 23 (*Cmklr1*, **E**), and a serotonin-related gene such as serotonin transporter (*Slc6a4*, **F**) were measured by RT-qPCR in PVAT from SS and SR rats fed a CD or HFD for 12 weeks. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's post hoc multiple comparisons test.

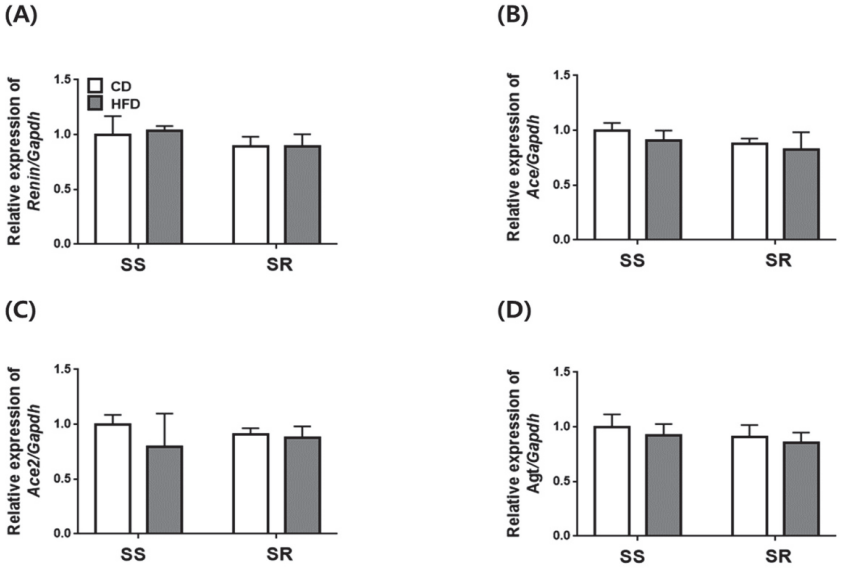

Figure 6. The effects of a high-fat diet on the expression of genes related to angiotensin II in kidneys from SS and SR rats. The mRNA expressions of angiotensin II-related genes such as *Renin* (**A**), *Ace* (**B**), *Ace2* (**C**), *Agt* (**D**) were measured by RT-qPCR in the kidneys of SS and SR rats fed a CD or HFD for 12 weeks. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's post hoc multiple comparisons test.

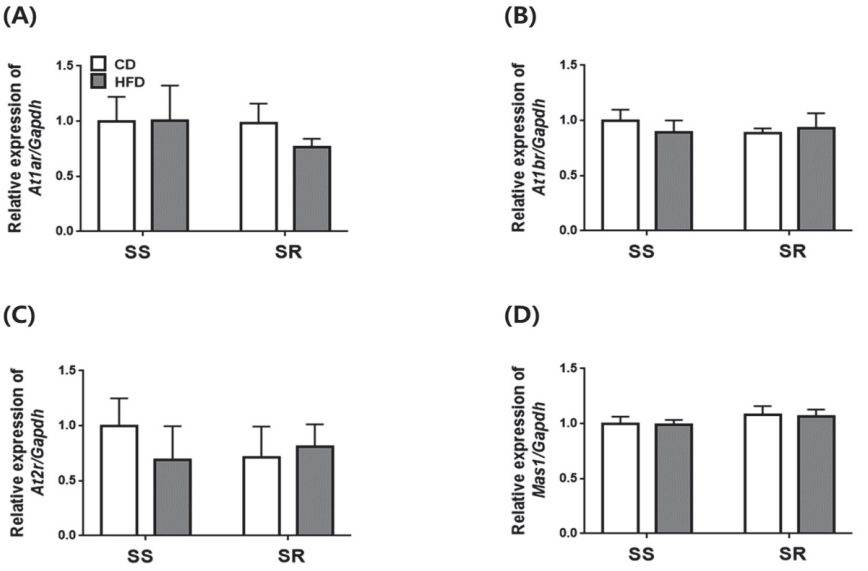

Figure 7. The effects of a high-fat diet on the expression of genes related to angiotensin II receptor in kidney from SS and SR rats. The mRNA expression of angiotensin receptor related genes such as *angiotensin II type 1a receptor* (*At1ar*, **A**), *angiotensin II type 1b receptor* (*At1br*, **B**), *angiotensin II type 2 receptor* (*At2r*, **C**), and *mas1 proto-oncogene* (*Mas1*, **D**) were measured by RT-qPCR in kidney from SS and SR rats fed a CD or HFD for 12 weeks. Graph, mean ± SEM of 6 independent experiments. Two-way analysis of variance followed by Tukey's post hoc multiple comparisons test.

4. Discussion

These results of this study demonstrate that SR rats are protected against hypertension during diet-induced obesity. We found that a HFD did not induce hypertension in SR rats, unlike in SS rats, although it increased both body weights and tPVAT weights. The HFD did not affect the mRNA expression of adipocyte markers or contractile factors in tPVAT of the SS and SR rats. There were no significant differences in the mRNA levels of RAAS genes in the kidneys of SS and SR rats before and after the HFD.

Salt sensitivity is defined as a decrease in mean arterial blood pressure of >5 mmHg during a low-sodium intervention or an increase of >5 mmHg during a high-sodium intervention, whereas salt resistance is defined as a change in mean arterial blood pressure of <5 mmHg during low-sodium or high-sodium interventions. The Genetic Epidemiology Network of Salt Sensitivity (GenSalt) study conducted in rural northern China demonstrated that ~32.4% of Chinese adults are sodium sensitive [21]. Physical activity and dietary potassium intake were associated with reduced sodium sensitivity, whereas older age, female gender, Black race, obesity, metabolic syndrome, and elevated BP were associated with increased sodium sensitivity [21]. In 65% of individuals with high blood pressure, the amount of salt intake had a greater effect on blood pressure in patients with metabolic syndromes such as hypertension, diabetes, and obesity than in those without the syndrome [22]. This model showed similar results in a previous study in that SS rats but not SR rats developed hypertension after high-fructose intake [19]. Similarly, SBP increased in spontaneous hypertensive rats (SHRs) after 12 weeks of a HFD, whereas SBP did not begin to increase in Wistar–Kyoto rats (WKY) until 12 weeks of a HFD [23]. In the present study, we characterized the development of hypertension in SS and SR rats by monitoring SBP, DBP, body weight, and food intake during a HFD (Figure 1). The HFD increased the SBP in the SS but not in the SR rats, although the HFD caused diet-induced obesity and tPVAT enlargement in both rats. Therefore, SR rats have a unique trait in that they are resistant to the development of diet-induced hypertension.

At a given renal perfusion pressure, the SR rats had higher renal blood flow with lower resistance than the SS rats did [24] because signaling via NO is more dominant than that via O2− in the thick ascending limb cells of the renal tubule [25]. However, in the SS rats, signaling via O2− is more dominant than that via NO in the thick ascending limb cells of the renal tubule, which results in higher vascular resistance with constriction of the renal blood vessels. Because the genetic traits that determine salt resistance in SR rats may also determine fructose or fat resistance, it remains to be investigated which genes of the SR rats determine salt resistance.

Obesity-induced hypertension has been linked to increased coagulability, endothelial dysfunction, and inflammation [26], as well as more conventional risk factors for cardiovascular diseases such as insulin resistance, hypertension, and atherogenic dyslipidemia consisting of hypertriglyceridemia, high low-density lipoprotein cholesterol particles, and suboptimal high-density lipoprotein cholesterol levels [27]. Hypertension mediates abnormal kidney function and vascular pathology. The mediators are physical compression of the kidneys by fat in and around the kidneys, activation of the RAAS, and increased activity of the sympathetic nervous system [28]. Obesity is associated with volume expansion of extracellular fluid, which increases blood flow in many tissues and, in turn, increases venous return and cardiac output [29]. Therefore, obesity is associated with functional vasodilation that is probably due to the increased metabolic rate and higher tissue oxygen consumption [28].

Because visceral fat and PVAT increase the risk for cardiovascular diseases [30], an alteration in body fat distribution might have a significant impact on high blood pressure. In states of obesity, as the PVAT mass increases, the contractile and the proinflammatory cytokine TNFα increase, whereas anticontractile factors significantly decrease [31]. We observed no significant differences between the SS and SR rats fed a CD or HFD in the mRNA expression of angiotensin II-related genes such as *Ace* and *angiotensinogen*, a norepinephrine-related gene such as *tyrosine hydroxylase*, chemerin-related genes such as *Rarres2* and *Cmklr1*, or a serotonin-related gene such as *Slc6a4* in tPVAT (Figure 5). Although there were no significant differences in the levels of the proinflammatory cytokines IL-6 and TNFα, the anti-inflammatory cytokine adiponectin was higher in the SR rats than in the SS rats fed a CD (Figure 4). PVAT-derived factors such as IL-6 [32] and TNFα [33] had a contractile or an anticontractile effect depending on the microenvironment. Regardless of whether the rats were fed a CD or HFD, there were no significant differences in the mRNA expression or cytokine levels in the SS and SR rats. Therefore, the factors with anticontractile or contractile effects may exert a contractile effect in obese SS rats.

An HFD tends to increase urinary protein excretion with marginal kidney damage and further accelerates salt-induced proteinuria and renal histological aggravation [34]. Although several studies have reported that HFD-induced hypertension increases the expression of RAAS in the kidneys of rodent models [35,36], no differences were found in the expression of the RAAS components (*Renin, Ace, Ace2, Angiotensinogen, At1ar, At1br, At2r,* and *Mas1*) in kidneys of SS and SR rats fed a CD or HFD. The increase in SBP did not correlate with the mRNA expression of the RAAS components in the kidney; these findings suggest that the mRNA expression of the RAAS components did not affect the BP in the SS and SR rats. Another study demonstrated that a HFD for 12 weeks did not induce a definite diabetic state despite hypertension in SHRs and suggested that a HFD for 24 weeks induced definite dyslipidemia and insulin resistance as well as systemic RAAS activation [12].

In particular, Nox2 represents endothelial and vascular dysfunction in metabolic disease and hypertension [37]. HFD-induced hypertension shows that endothelial Nox2-derived superoxide plays a critical role in endothelial dysfunction, whereas Nox2 deficiency or pharmacologic Nox2 inhibition protects against vascular oxidative stress. Nox1 and Nox2 primarily produce $O_2^{\cdot -}$. Nox4 is a functional source of reactive oxygen species generation in the mitochondria of the kidney cortex, wherein mitochondrial superoxide dismutase (SOD) effectively dismutates Nox4-derived superoxide to H_2O_2 [38]. In our

study, *Nox2* was markedly higher in the kidney of SR rats than in SS rats, with no significant changes in *Nox1*, *Nox3*, or *Nox4* (Figure S1).

The constriction of the mesenteric vascular bed provides an important contribution to the total peripheral resistance [39]. Structural or functional alterations in the mesenteric vascular bed contribute to the hypertensive process [40]. The mesenteric arteries of SHR express a greater density of calcium channels than those of WKY, which exhibited exaggerated constrictor responses to a variety of stimuli in SHR [41,42]. A high-salt diet was shown to decrease SOD activity in the mesenteric arteries of SS rats [43]. An HFD induced endothelial dysfunction in the mesenteric vascular bed due to a proinflammatory and contractile state of the PVAT [44].

This study has some limitations. First, we measured SBP and DBP by the tail-cuff method. Second, PVATs at different anatomical locations present different phenotypes [1]. The brown adipose tissues in the thoracic aorta were not whitened by diet-induced obesity/inflammation and did not have the characteristics of white adipocytes [45,46]. Although the HFD increased the body weights and tPVAT weights as well as adipocyte areas of the rats, it did not affect the properties (Figures 2 and 3). Although SS rats had similar cellularity in white and brown adipocytes as compared to SR rats, SS rats had higher cellularity in beige adipocytes than SR rats, which were not affected by the HFD. It would be of interest to determine whether an HFD affects abdominal or mesenteric PVAT, as the mesenteric vascular bed plays a key role in blood pressure regulation. Finally, although the HFD groups and CD groups had a similar calorie intake, it is possible that reduced energy expenditure contributed to increases in body weight in the HFD groups [17].

In summary, the blood pressure of the SR rats fed an HFD was not affected by the fat accumulation, properties of tPVAT, or RAAS in the kidney. Therefore, our results indicate that the SR rat is protected against hypertension during diet-induced obesity. This finding implies that the genetic trait that determines salt sensitivity may also determine fructose and fat sensitivity and that it is associated with the prevention of hypertension.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14183843/s1, Figure S1: The effects of a high-fat diet on the expression of genes related to nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) in kidneys from SS and SR rats; Table S1: Primers for RT-qPCR.

Author Contributions: I.K. conceived the study and attained the funding; S.L. and S.J. developed and designed the experiments; S.L., S.J., and J.Y.K. performed the experiments; S.L. and S.J. analyzed the data; S.L. wrote the paper which was revised critically by I.K. for important intellectual content. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (NRF-2021R1A2B502001763, and 2021R1A4A1021617), and a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI15C0001).

Institutional Review Board Statement: The Institutional Review Board of Kyungpook National University approved the care and use of animals (approval No. 2021-0192). Every effort was made to minimize both the number of animals used and their suffering. SS rats (DIS/EisSlc; Dahl-Iwai S) and SR rats (DIR/EisSlc; Dahl-Iwai R) were purchased from Japan SLC, Inc. (Hamamatsu, Shizoka, Japan).

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

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

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

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nutrients

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Review

Can Fasting Curb the Metabolic Syndrome Epidemic?

Josip Vrdoljak [1], Marko Kumric [1], Marino Vilovic [1], Dinko Martinovic [1], Veljko Rogosic [2,3], Josip A. Borovac [1,4,5], Tina Ticinovic Kurir [1,6] and Josko Bozic [1,*]

[1] Department of Pathophysiology, University of Split School of Medicine, 21000 Split, Croatia; josip.vrdoljak@mefst.hr (J.V.); marko.kumric@mefst.hr (M.K.); marino.vilovic@mefst.hr (M.V.); dinko.martinovic@mefst.hr (D.M.); jborovac@mefst.hr (J.A.B.); tticinov@mefst.hr (T.T.K.)
[2] Department of Ophthalmology, University of Split School of Medicine, 21000 Split, Croatia; veljko.rogosic@mefst.hr
[3] Department of Ophthalmology, University Hospital of Split, 21000 Split, Croatia
[4] Department of Health Studies, University of Split, 21000 Split, Croatia
[5] Department of Cardiology, University Hospital of Split, 21000 Split, Croatia
[6] Department of Endocrinology, Diabetes and Metabolic Diseases, University Hospital of Split, 21000 Split, Croatia
* Correspondence: josko.bozic@mefst.hr

Abstract: Metabolic syndrome (MetS) represents a cluster of metabolic abnormalities that includes hypertension, central obesity, insulin resistance, and atherogenic dyslipidemia. Due to the high prevalence (around 1/3 of the world population) economic burden of MetS, there is a need for new dietary, lifestyle, and therapeutic options. Recently, fasting emerged as a dietary method proposed for controlling metabolic risk factors. Intermittent fasting (IF), or time-restricted feeding (TRF), describes an array of feeding patterns in which calorie intake is restricted to a specific time period. Hence, this review aimed to elucidate the latest data on MetS and explore the viability of simple management options, such as IF and TRF. Preclinical studies have shown how IF/TRF exerts beneficial effects on the gut microbiota, glucose and insulin metabolism, weight and visceral fat, and lipid metabolism. However, the results obtained from human studies are somewhat conflicting, as weight loss was achieved in all studies, whereas in some studies, there was no significant effect on insulin resistance, cholesterol/lipid metabolism, or blood pressure. Nevertheless, as only very few human studies were performed, there is a need for more randomized control trials on larger cohorts of patients with MetS to gather higher-yield evidence to clarify whether IF/TRF are suitable dietary patterns for this population.

Keywords: metabolic syndrome; fasting; intermittent fasting; time restricted feeding; diabetes mellitus; hypertension; dyslipidemia; obesity

Citation: Vrdoljak, J.; Kumric, M.; Vilovic, M.; Martinovic, D.; Rogosic, V.; Borovac, J.A.; Ticinovic Kurir, T.; Bozic, J. Can Fasting Curb the Metabolic Syndrome Epidemic?. *Nutrients* **2022**, *14*, 456. https://doi.org/10.3390/nu14030456

Academic Editors: Abeer M. Mahmoud and Shane Phillips

Received: 16 December 2021
Accepted: 19 January 2022
Published: 20 January 2022

1. Introduction

Central obesity, elevated blood pressure, dyslipidemia, and insulin resistance are cardiometabolic risk factors grouped together in metabolic syndrome (MetS). Since the WHO defined MetS in 1999, there have been multiple other definitions of MetS, with the most recent harmonized definition [1,2]. All components of MetS are plaguing the world with an increasing incidence and prevalence [3–5]. Obesity, mostly of the central type, is a pathophysiological cornerstone of MetS and is considered to be the main culprit that leads to other metabolic disturbances found in MetS [4,6–8]. Epidemiological studies have shown that in the last 40 years, obesity prevalence has doubled, to such a degree that nearly a third of the world's population is now considered overweight or obese [5,9]. Before the start of the globalization trend, obesity was most prevalent in the US and the countries of Western Europe, while currently we are witnessing an increase in obesity rates in all ages and sexes, regardless of geographical location, socioeconomic status, and ethnicity [5,10]. Likewise, the prevalence of MetS in the US is also high, at 34.7% (95% CI, 33.1–36.3%) [11].

Similar trends in prevalence are also found in other countries across the globe, with the highest MetS prevalence in Mexico (41%) [12–15].

Furthermore, the prevalence of hypertension is also on the rise, particularly in low-middle income countries [16–18]. In addition, the same is true for the prevalence of insulin resistance/diabetes mellitus type 2 and dyslipidemia [19,20]. The cost of treating these comorbidities is substantial. According to the American Diabetes Association (ADA), the total costs of diagnosed diabetes have risen from $245 billion in 2012 to $327 billion in 2017, resulting in a 26% increase over a five-year period [21]. Moreover, adults with obesity in the US experienced $2505 higher annual health care costs, which is a 100% higher cost paid compared to those with lesser weight [22]. Similarly, according to American Heart Association (AHA) and Medical Expenditure Panel Survey (MEPS) data from (2016–2017), the costs of treating cardiovascular disease (CVD) in the US were estimated to be $363.4 billion (or $406 billion, inflation-adjusted) [23].

There are no studies directly examining the economic burden of MetS per se. Still, since other studies have shown how the prevalence of MetS among obese adolescents and adults ranges from 30.3% up to 52%, we may postulate that the direct medical costs of MetS are around 1/3 to 1/2 of the costs of obesity (from $78 billion to $135 billion) in the US [24,25]. Since MetS and obesity mostly precede CVD and type II diabetes, there is a growing need for cheap and effective treatment strategies to combat this increasing burden.

Lifestyle and dietary changes (physical activity, calorie intake/bodyweight reduction) are at the forefront of metabolic syndrome treatment [26–28]. One of the dietary methods proposed for controlling metabolic risk factors is fasting [29]. Intermittent fasting (IF) and time-restricted feeding (TRF) describe an array of feeding patterns in which calorie intake is restricted to a specific time period (8 h feed/16 h fast, 4 h feed/20 h fast, alternate day fasting, etc.) [30]. Recently, IF/TRF has shown promising results in managing cardiometabolic risk factors [29,31,32].

Hence, in the first part of this comprehensive review, we present the latest data on MetS epidemiology and pathophysiology, while in the second part, we explore the viability of IF and TRF for managing MetS.

2. The Definition of Metabolic Syndrome

In 1988, during his Banting lecture, Reaven characterized a cluster of conditions related to insulin resistance. He called the cluster "Syndrome X" or "the deadly quartet", and it consisted of obesity, non-insulin-dependent diabetes mellitus, hypertension, and dyslipidemia [26,33]. Since then, multiple definitions have been brought forward to better encompass this clustering of cardiometabolic risk factors. These include the WHO definition from 1998, the NCEP (National Cholesterol Education Program) definition from 2003, and the IDF (International Diabetes Federation) definition from 2006 [20]. To avoid further discrepancies and to standardize the diagnostic criteria, several major health organizations jointly produced the harmonized definition of MetS that is currently in use [34].

According to the harmonized definition, MetS is defined by the following criteria:

(1) The presence of insulin resistance/prediabetes (glucose level > 100 mg/dL (5.6 mmol/L)), or diagnosed type 2 DM.

(2) Enlarged waist circumference (the exact values of which are adjusted according to population-specific and country-specific criteria).

(3) HDL-C < 40 mg/dL (1.03 mmol/L) in men and <50 mg/dL (1.29 mmol/L) in women or triglycerides $\geq$ 150 mg/dL (1.69 mmol/L) (with the inclusion of those taking medicine to treat dyslipidemia).

(4) Systolic blood pressure $\geq$ 130 mm Hg or diastolic blood pressure $\geq$ 85 mm Hg (including patients on anti-hypertensive therapy).

3. Epidemiology of Metabolic Syndrome

The central type of obesity is the most common characteristic found in MetS; therefore, the incidence and prevalence of MetS closely follow that of obesity [35,36]. Every two

years, the US conducts the National Health and Nutrition Examination Survey (NHANES), in which they obtain obesity rates among people aged two or older. The latest data for 2017–2018 show that obesity prevalence among adults was 42.4% [37]. By contrast, the obesity rates for adults at the start of the decade were 35.7%, with a rising trend of approximately 2% every two years. The rising trend is slower for the pediatric population, with the 2009–2010 obesity rate at 16.9% and the 2017–2018 obesity rate at 19.3% [37]. In a study by Hirode et al., where the authors examined NHANES data form 2011 to 2016, among 17048 participants, the MetS weighted prevalence was 34.7% (95% CI, 33.1–36.3% [n = 5885]) [11]. In other words, as much as one-third of the US adult population suffers from MetS.

The large prevalence is not restricted to the US, as comparable data were found in Brazil, where the latest MetS prevalence was 38.4% [13]. High waist circumference (65.5%) and low HDL cholesterol (49.4%) were the most prevalent MetS components among the Brazilian population. In addition, MetS was more frequent among women (41.8%), individuals with less education (47.5%), and older adults (66.1%) [13]. Similarly, in Mexico, investigators performed a systematic meta-analysis on 15 studies in which the pooled prevalence of MetS was 41% (95% CI 0.34–0.47) [12].

In Asia, a metanalysis in which investigators pooled MetS prevalence data from the Chinese population from 2008–2015 found that the pooled prevalence for subjects aged 15 years and older was 24.5% (95% CI: 22.0–26.9%). This metanalysis on the Chinese population also saw a similar trend, according to which the MetS prevalence was higher in females 27.0% (95% CI: 23.5–30.5%) vs. males 19.2% (95% CI: 16.9–21.6%) [38]. However, another study on the Chinese population showed marked differences in MetS prevalence between various ethnic groups. The Korean population featured the highest MetS prevalence (35.42%), the Hui population the second highest (22.82%), while the Mongolian and Tibetan populations featured the lowest (11.61%) and (6.17%) respectively [39].

Furthermore, in a European study examining data from two cohorts, one from Russia and the other from Italy, the MetS prevalence was 37% for the former and 21% for the latter [40]. In addition, another study examined the data from 34,821 subjects from 12 cohorts from 10 European countries and one cohort from the USA. MetS prevalence was 24.3% (8468 subjects: 23.9% in men vs. 24.6% in women, $p < 0.001$), with an age-related increase in prevalence across all cohorts [15]. Furthermore, in a study on the Portuguese population, in which data were gathered from 2007 to 2009, MetS prevalence was 36.5%, 49.6%, and 43.1%, using the Adult Treatment Panel III, International Diabetes Federation, and Joint Interim Statement definitions, respectively [14]. MetS prevalence was significantly higher in women and the older population in Portugal, as shown in the aforementioned studies. At the same time, it was also more frequent in non-urban areas than in urban areas ($p = 0.001$) [14]. Interestingly, in contrast to these findings, a study on the Czech population found that MetS is less common in females 25.5%, then in males 37.6% [41]. Akin to these findings, MetS prevalence was also higher in Slovakian males (30.2%), than in females (26.6%), with an increasing trend from 2003 to 2012 [42].

When examining the data we mentioned, we can see large differences in MetS prevalence among various populations, from the low of 11.61% in the Tibetan population, to the high of 41% in the Mexican population [12,39]. The reasons behind the observed discrepancies are probably a result of different lifestyles, with Western dietary habits making people more susceptible to MetS. If we take a closer look at migrant populations (from countries with low to countries with high rates of obesity) we observe a rather interesting twist. Namely, although migrants arrive with a health advantage, including generally healthier body weight, intrinsic and environmental factors combine to cause unhealthy weight gain, often to beyond the levels seen in native populations [43]. According to Neel et al., this observation may be explained from an evolutionary standpoint. Although the loss of uricase may have provided a survival advantage by amplifying the effects of fructose to enhance fat stores, and by increasing blood pressure in response to salt, the absence of the "thrifty" uricase gene may have caused a range of detrimental metabolic effects on modern

humans (characterized by excessive caloric intake), thus explaining the current epidemic of obesity and diabetes [44]. The putative mechanism associating fructose and loss of uricase lies in the observation that uric acid may regulate fructose metabolism by affecting fructose transporters in the intestines and fructokinase in the liver [45]. Nevertheless, with the Western lifestyle prevailing in most of the world, there is an increasing global trend of MetS prevalence, with approximately one-quarter of the world's population currently suffering from MetS [11,20,46].

4. Pathophysiological Background of Metabolic Syndrome

As is the case with other chronic non-communicable diseases, MetS also results from a complex interplay between genetic and environmental factors. Currently, central obesity/visceral adipose tissue (VAT) is considered to play one of the main roles in initiating the deadly quartet of MetS. VAT exerts its influence on glucose and lipid metabolism via multiple mechanisms. Firstly, VAT is a major source of free fatty acids (FFA), which are directly connected to the liver via splanchnic circulation [4]. In the liver, FFAs lead to increased gluconeogenesis, as well as increased triglycerides and very low-density lipoprotein (VLDL) production [6]. The increase in liver FFA oxidation induces a decrease in xylulose 5-phosphate, which results in the activation of gluconeogenesis (by inhibiting phosphofructokinase 1 and activating fructose-1,6-bisphosphatase) [47]. Furthermore, the ectopic accumulation of lipid metabolites (ceramides, diacylglycerol, acetyl-CoA and fatty acids) decreases insulin sensitivity [48]. These lipid metabolites, in turn, activate serine/threonine kinases (protein kinase C (PKC), nuclear factor-kB (NFkB), inhibitory kB kinase b (IKKb)), which then phosphorylate insulin receptor substrate (IRS) and protein kinase B/Akt, and therefore inhibit insulin signaling [47,48].

Moreover, adipose tissue is a source of many cytokines and hormones, called adipokines. Studies have shown that central obesity/VAT is related to dysregulated adipokine secretion, with increased levels of plasminogen activator inhibitor (PAI-1), tumor necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), angiotensinogen, and interleukin 6 (IL-6). In addition, leptin, a hormone that regulates satiety, energy expenditure, and appropriate glucose homeostasis, is directly correlated to the amount of white adipose tissue. Even though, in physiological conditions, leptin promotes satiety and signals the amount of fat storage to the hypothalamus, it seems that in MetS, there is a leptin resistance or a certain ceiling on the possible effect of leptin, beyond which new leptin stimulates little effect [49].

On the other hand, in central obesity, there are decreased levels of adiponectin, which is considered the "good" adipokine. The primary action of adiponectin is phosphorylation and the activation of key intermediates in the insulin signaling pathway, increasing insulin sensitivity [50]. Therefore, a lack of adiponectin in MetS promotes insulin resistance and disrupts glucose homeostasis.

The aforementioned TNF-α, IL-6, and MCP-1 are pro-inflammatory cytokines, which contribute to the systemic low-grade inflammation found in MetS. At the same time, PAI-1 increases the risk of thrombosis and accelerates the development of atherosclerosis [4,51]. This low-grade inflammation leads to further insulin resistance in muscles, as well as to disruption of o β-cells [52,53].

As recently reviewed, there is also essential gut–adipose tissue crosstalk, which is disrupted in the setting of MetS. Postprandial incretins, glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic peptide (GIP), which regulate glucose homeostasis and exert anorexigenic effects, are significantly decreased in obese or T2DM patients [54]. Furthermore, in patients with obesity and MetS, the levels of appetite-inducing hormone ghrelin fail to progressively decline after meal ingestion [54]. This disruption in anorexigenic/orexigenic hormone homeostasis induces a positive loop that ultimately ends in obesity/MetS.

Finally, the pathogenesis of hypertension in MetS is multifactorial as well. Hyperinsulinemia exerts an anabolic effect on the heart muscle and the media of the blood

vessel wall. It also promotes sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS) activity, leading to vasoconstriction, sodium retention, and endothelial dysfunction [55]. Interestingly, recent studies have also elucidated leptin's role in obesity-related hypertension. Acting on its receptors in the hypothalamus, leptin initiates a downstream signal transduction that ends in the preganglionic autonomic neurons of the spinal cord, leading to increased sympathetic activity in the kidneys and, therefore, increased blood pressure [56]. Nevertheless, each presented mechanism's relative contribution to hypertension development remains elusive.

Moreover, the presence of obstructive sleep apnea and baroreflex dysfunction in MetS further increase SNS activity [57–59].

While the current understanding of MetS pathophysiology is discussed above, it is a continuously improving subject with new research that will help us better understand the puzzle of MetS.

5. Effects of Diet on Metabolic Syndrome

The Western diet, characterized by a high intake of red and processed meat, refined grains, sweets, and sugary beverages, is associated with an increased risk of developing MetS [60,61]. This diet is calorie-dense, rich in small-chain fatty acids (SFA), simple carbohydrates, and other nutrients that feature pro-inflammatory properties, disrupt the gut microbiota, and dampen insulin sensitivity [61–63]. A meta-analysis by Fabiani et al. has shown that the "Meat/Western" pattern leads to a 19% increase in MetS risk, while a "Healthy" dietary pattern (fruit, vegetables, whole grains, fish, no processed food/high content of vitamins, minerals, antioxidants, fiber, MUFA, and n-3 fatty acids) is associated with a 15% decrease in MetS risk [60]. Similar results were obtained in another meta-analysis that also studied the relationship between a posteriori dietary patterns and MetS: a healthy/prudent diet was associated with a lower prevalence of MetS, while an unhealthy/Western pattern was associated with an increased risk of developing MetS [64]. Another popular dietary pattern, the Mediterranean diet, has also shown benefits regarding MetS [65,66]. In a meta-analysis by Kastorini et al., the combined effect of prospective studies and clinical trials showed that the Mediterranean diet is associated with a reduced risk of MetS (log hazard ratio: −0.69, 95% confidence interval (CI): −1.24 to −1.16) [67]. Comparable results were achieved with the dietary approaches to stop hypertension (DASH), where multiple studies showed how the DASH diet led to a reduction in systolic and diastolic blood pressure, a reduction in BMI and waist circumference, an improvement in cardiometabolic profile, and a reduction in T2DM incidence [68–72].

Therefore, there is substantial evidence that diets such as the Mediterranean diet and DASH exert a beneficial effect on cardiometabolic risk factors, with a common theme in which foods such as vegetables, fruit, whole grains, and fish are associated with these benefits. Nevertheless, in addition to the diet itself, dietary regime adjustments may provide metabolic benefits regardless of the amount and type of food ingested.

6. Metabolic Syndrome and Fasting

6.1. Evidence on Animal Models

A number of studies have exhibited the beneficial effects of fasting/time-restricted feeding on animal models. A number of studies have exhibited the beneficial effects of IF/TRF on animal models. In a study by Hatori et al., the authors demonstrated the favorable effects of TRF in mice fed with a high-fat diet (HFD, 61% energy from fat). The group fed during an 8 h period lost weight and exhibited better insulin sensitivity, less hepatosteatosis, less inflammation, and improved motor coordination while consuming the same number of calories as the group fed ad libitum (AL) [73]. The beneficial effect of TRF was substantiated from a biochemical standpoint as well, since the TRF regimen improved CREB, mTOR, and AMPK (metabolic master regulator) pathway function [73]. Similar results were achieved in another study where, in comparison to mice on high-fat (HF) AL diets, mice on a time-restricted HF diet experienced an 18% reduction in body weight, a

30% reduction in cholesterol levels, 10% lower TNF-α levels, and a 3.7 fold increase in insulin sensitivity [74]. Moreover, in the same study, the authors compared mice on a time-restricted HF diet with mice on an AL low-fat diet consuming the same number of calories. In this comparison, the TRF mice showed a 2% bodyweight reduction, a 21% reduction in cholesterol levels, and a 1.4 fold increase in insulin sensitivity [74].

Furthermore, in a recent study, Pak et al. used a series of feeding regimens to dissect the effects of caloric restriction (CR) and fasting [75]. Four groups of mice were examined: (1) mice given AL access to a regular rodent diet; (2) mice provided with AL access to chow that was 50% diluted with indigestible cellulose (corresponding to a 30% CR); (3) mice fed 30% less food than AL-fed mice using an automatic feeder to release food in three equal portions during the 12 h dark period; (4) mice fed once per day in the morning, with 30% restriction compared to the AL group; (5) mice that were trained to consume approximately the same quantity of the food as AL-fed mice, but within three hours. The crucial comparison was between mice on a diet according to which they consumed fewer calories without imposed fasting (AL chow diluted with 50% indigestible cellulose) and mice that were fed only once per day in the morning (without CR). The study showed that fasting is needed for CR-induced improvements in glucose metabolism, frailty, and lifespan in C57BL/6J male mice. In addition, the results elucidate how fasting alone, without the reduced intake of calories, is sufficient to realize the metabolic phenotypes and transcriptional signature of a CR diet [75]. The importance of these studies that they show the benefits of TRF whilst controlling for caloric intake, thus decoupling the effects from caloric restriction.

Other studies have proven the benefits of fasting in alleviating circadian clock disruptions and improving metabolic homeostasis [29,76]. As reviewed in a paper by Świątkiewicz et al., a proper TRF cycle sustains circadian rhythms and restores normal daily rhythms in many mRNAs, proteins, and metabolites that are involved in the homeostasis of carbohydrates and lipids [29]. TRF also regulates circulating leptin and adiponectin levels, and exerts multiple valuable effects on the main organs involved in metabolic homeostasis (liver, muscle, white and brown adipose tissue, gut, and brain) [29,76–78]. Interestingly, a study on mice has shown the effects of IF (every-other-day fasting – EOFD) on white adipose tissue browning [79]. The EOFD mice exhibit significantly more beige fat development within white adipose tissue, were less obese, better insulin sensitivity, and less hepatic steatosis. The researchers also pointed out how these beneficial effects are most probably exerted through the shaping of gut microbiota because the microbiota-depleted mice were resistant to EODF-induced beiging, while microbiota transplantation from EODF-treated mice to microbiota-depleted mice activated beiging and improved metabolic homeostasis [79]. Moreover, a study on a drosophila melanogaster (fruit fly) model of obesity, in which flies were subjected to a 12 h TRF, exhibited improved muscular function (fewer intramuscular fat deposits), improved phosphorylated AKT levels, fewer mitochondrial aberrations, and better insulin sensitivity [80].

Importantly, not all fasting protocols exhibit beneficial effects, as was shown in a study investigating alternate-day fasting in young female Wistar rats (24 h fasts intercalated with 24 h of free access to the same chow) [81]. Alternate-day IF decreased weight gain and food intake, but it led to increased fat reserves, elevated plasma insulin concentrations, and reduced muscle mass. Although the study was conducted on young and healthy animals with no MetS, these findings suggest the potential adverse effects of alternate-day fasting and promote caution in thinking that all fasting methods are similar and beneficial.

Overall, the above-noted animal studies have set the biochemical and pathophysiological evidence and groundwork (positive effects on weight, obesity, gut microbiota, energy metabolism, and circadian rhythms) for further human studies on patients with obesity and MetS (Figure 1, Table 1).

Table 1. Studies examining fasting on animal models of MetS and obesity.

Study	Cohort	Fasting Regime	Duration	Results
Hatori et al. [73]	Mice fed with a high-fat diet (HFD, 61% energy from fat)	16 h fast/ 8 h eating window	18 weeks	↔ caloric intake ↓ body weight ↑ insulin sensitivity ↓ hepatosteatosis ↓ inflammation ↑ motor coordination ↑ CREB, mTOR, AMPK function
Sherman et al. [74]	Mice fed HFD ad libitum/mice fed HFD time-restricted	Fed during zeitgeber time 4 and 8 (zeitgeber time 0 is the time of lights on)	18 weeks	↓ body weight ↓ cholesterol levels ↓ TNF-α ↑ insulin sensitivity
Pak et al. [75]	Ad libitum mice/30%CR mice/30%CR in 12 h feed period/30% CR mice fed once in morning/ Ad libitum mice in 3-h feed period	12 h TRF/ Once in the morning feeding/ 3 h TRF	16 weeks	Fasting is needed for CR-induced improvements in glucose metabolism, frailty, and lifespan in C57BL/6J male mice
Li et al. [79]	Mice fed ad libitum chow diet/ mice on EODF	Every-other-day fasting (EODF)	30 days	↑ white adipose tissue browning ↓ obesity ↓ hepatic steatosis ↑ insulin sensitivityeffects exerted through shaping of the gut microbiota
Villanueva et al. [80]	Drosophila melanogaster (fruit fly) model of obesity	12 h TRF	3 weeks	↓ muscular fat deposits ↑ Phospho-AKT level ↑ insulin sensitivity ↓ mitochondrial aberrations
Munhoz et al. [81]	Young female Wistar rats	24 h fast/ 24 h of free access to the same chow	12 weeks	↓ weight gain ↓ food intake ↑ fat reserves ↑ plasma insulin concentrations ↓ muscle mass

Abbreviations: HFD = high-fat diet, CR = caloric restriction, CREB = cAMP response element-binding protein, mTOR = mammalian target of rapamycine, AMPK = 5′ AMP-activated protein kinase, TNFα = tumor necrosis factor alpha, TRF = time-restricted feeding, EODF = every-other-day fasting.

6.2. Evidence in Human Studies

Currently, there is little research on the effects of fasting on patients with diagnosed MetS [29,31]. However, several small-scale and pilot trials on obese patients and patients with cardiometabolic risk clustering (but no MetS diagnosis) have provided promising results [29,31,82]. Similarly, a recent systematic review on the effects of fasting on cardiometabolic risk factors supports the role of IF in improving metabolic health [83]. Small but significant improvements were detected in risk factors such as body weight, waist circumference, fat mass, BMI, blood pressure, total cholesterol, triglycerides, fasting blood glucose, fasting insulin, and insulin resistance [83].

On the other hand, there are also some contradictory results, as one randomized control trial on obese or overweight adults produced only modest weight loss (below 2% of initial body weight), but no real benefits of IF on metabolic parameters or fat loss in the absence of controlled food intake [84].

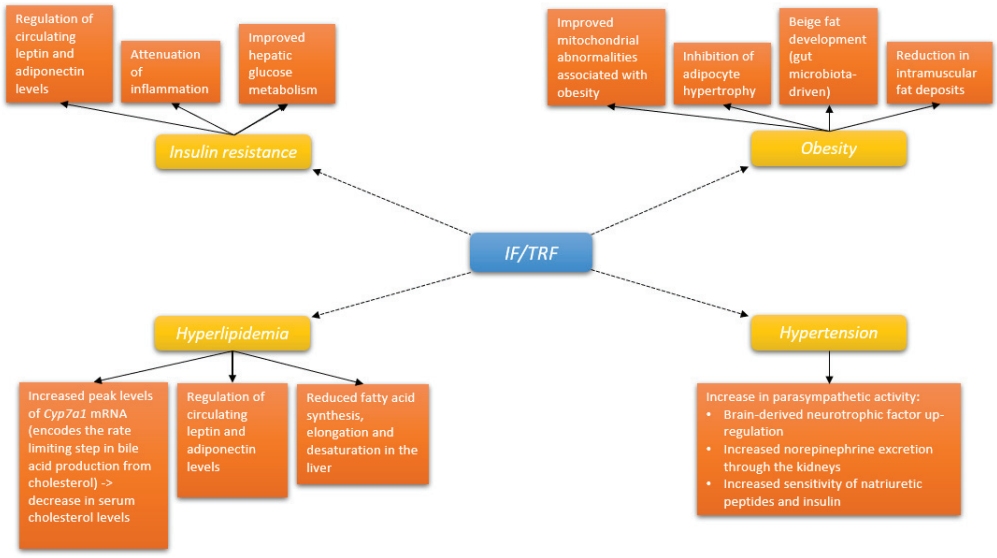

Figure 1. Pathways through which intermittent fasting/time restricted feeding may affect the constituents of metabolic syndrome. Abbreviations: IF: intermittent fasting; TRF: time-restricted feeding.

A single-arm, paired-sample trial by Wilkinson et al. investigated the effects of 14 h fasts for 12 weeks on 19 patients with MetS who received the standard of care (antihypertensive therapy and/or statin) [31]. To track caloric intake and the adherence to TRE intervention, a validated app—myCircadianClock (mCC) was used [31]. Although there were no recommendations to reduce caloric intake, an 8.62% decrease in mean daily caloric intake was recorded during the intervention. Significant bodyweight reduction from baseline was achieved (−3%), along with desirable reductions in body fat percentage (−3%), along with significant decreases in visceral fat rating (−3%) and waist circumference (−4%) [31]. Importantly, the authors also noted significant decreases in systolic and diastolic blood pressure, total cholesterol, LDL-C, and non-HDL-C [31]. Despite the study's limitations (it was an unblinded, single-arm pilot study with a relatively small sample size), it still provided valuable evidence on the safety, adherence, and probable efficacy of TRF in MetS management.

In a proof-of-concept study, Sutton et al. investigated the effects of 5 week early TRF (eTRF, eating period from 8 a.m. to 2 p.m.) in men with prediabetes, while controlling for weight loss (feeding the participants enough food to maintain their weight) [84]. In the study, eTRF reduced insulin levels, blood pressure, and oxidative stress, while it improved β-cell responsiveness and insulin sensitivity. Although the study was conducted on just eight participants, excellent adherence was achieved, since the participants were required to eat all meals under supervision [84,85]. The importance of this randomized controlled trial was that it demonstrated the benefits of IF independent of food intake and weight loss in humans.

In another study, researchers examined the effects of 8 h TRF over 12 weeks on 23 obese adults, compared to a matched historical control group [82]. There were significant decreases in body weight, energy intake, and systolic blood pressure in the TRF group ($-2.6\% \pm 0.5$; -341 ± 53 kcal/d; -7 ± 2 mm Hg, respectively). Interestingly, there were no significant differences in fat mass, lean mass, visceral fat mass, diastolic blood pressure, LDL cholesterol, HDL cholesterol, triglycerides, fasting glucose, fasting insulin, HOMA-IR, or homocysteine [82].

Kesztyüs et al. conducted a pilot study on the effects of TRF in abdominally obese participants (waist-to-height ratio, WHtR ≥ 0.5) in a general practitioner's office [86]. Forty

participants were asked to restrict their daily eating time to an 8–9 h window. On average, the participants reached the 15–16 h fasting target in 86 ± 15% of all recorded days. In addition, the participants achieved moderate weight loss (−1.7 ± 2.5 kg), along with a marked reduction in waist circumference (−5.3 ± 3.1 cm), leading to a reduction in WHtR (−0.03 ± 0.02) [86].

Moreover, a randomized controlled trial that researched the effects of two-month 4 and 6 h TRF in obese adults, resulted in promising outcomes concerning weight and cardiometabolic health [87]. Of 58 participants, 19 were randomized into the 4 h TRF group, 20 into the 6 h TRF group, and 19 into the no-intervention control group. Both the 4 and 6 h TRF regimens achieved a similar reduction in daily calorie intake (−550 kcal), while also producing similar weight loss (−3% body weight). Additionally, they reported a marked reduction in fasting insulin, insulin resistance, and oxidative stress. However, the significance of insulin and insulin resistance reductions was partly driven by a worsening in the control arm [87].

A trial by Parr et al. investigated the feasibility of TRF for individuals with T2DM [88]. The intervention consisted of a 2 week Habitual period to establish a baseline dietary intake, followed by a 6 week TRF intervention. Of the 24 enrolled participants, 19 completed the study. Overall daily dietary intake did not change between habitual and TRF periods. Moreover, the compliance with the 9 h TRF period was 72 ± 24% of 28 days (i.e., ~5 days/week). Interestingly, TRF did not significantly influence glycemic control or body mass, whereas the participants described hunger, daily stressors, and emotions as the main barriers to adherence. [88]. Furthermore, in a study by Chow et al., the authors examined and compared the effects of TRF (8 h target eating window) to an unrestricted (non-TRF) diet in overweight individuals [89]. Compared to non-TRF, the TRF group exhibited significantly reduced weight, lean mass, and visceral fat. Furthermore, when the TRF group was compared to their pre-intervention state, marked reductions in weight (−3.7%), fat mass (−4%), lean mass (−3.0%), and visceral fat (−11.1%) were observed. Interestingly, metabolic measures (lipids, blood pressure, 2 h oral glucose tolerance test, 2 week continuous glucose monitoring), and physical activity (actigraphy-assessed) remained unchanged [89]. The same research group performed a secondary analysis of the aforementioned trial, where they examined the effects of TRE on quality of life (QoL) measures [90]. TRE did not adversely affect QoL, and it even led to modest QoL improvements relative to baseline and unrestricted eating [90].

By contrast, in a prospective, randomized controlled trial conducted on 116 overweight or obese adults, Lowe et al. compared the effects of TRF (eating from 12–8 pm) with consistent meal timing (CMT-3 structured meals per day) over 12 weeks [84]. There was no recommendation for calorie and macronutrient intake or physical activity, so the study only compared the effects of different meal timing. There was a significant decrease in weight in the TRF group (−0.94 kg; 95% CI, −1.68 kg to −0.20 kg; $p = 0.01$), but no significant differences in weight change between groups (−0.26 kg; 95% CI, −1.30 kg to 0.78 kg; $p = 0.63$). There were no significant within-group or between-group differences in glucose and lipid metabolic parameters [84].

In a randomized controlled trial, Parvaresh et al. investigated the differences between a modified alternate-day fasting regime (ADF) (a very low-calorie diet, 75% energy restriction on Saturday, Monday, and Wednesday accompanied by 100% energy intake on Sunday, Tuesday, Thursday, and ad libitum feeding on Friday) and a standard caloric restriction diet (consuming 75% of energy needs each day) during an 8 week period [91]. Of 70 patients with MetS, 69 completed the study, and the analysis showed significant reductions in body weight, waist circumference, systolic blood pressure, fasting plasma glucose in the ADF group. Interestingly, there were no significant differences in triglyceride, cholesterol (total, HDL, LDL), HOMA- IR, or fasting insulin concentrations [91]. The strengths of this study are in the number of participants (69, compared to the 23 in the second-largest study on MetS/fasting) and its randomized design [31,91].

In summary, a common theme in all of the aforementioned studies is a significant reduction in body weight and waist circumference (Table 2). At the same time, there are mixed results regarding the improvement in insulin sensitivity, glucose and lipid homeostasis, and blood pressure. Interestingly, there are only two studies conducted on participants with diagnosed MetS. In addition to significant weight and waist circumference reduction, both studies also reported a significant reduction in blood pressure levels, albeit with mixed results in cholesterol reduction [31,91]. However, it is possible that the observed improvements using IF/TRF may not be sustained unless close monitoring and follow-up of adherence are performed. Therefore, a combination of patient education with dietary, medical, and coaching staff familiar with the IF protocol is critical in achieving the putative results. On the other hand, the potential downsides of IF/TRF that require attention are hypoglycemic reactions, cardiac arrhythmias, muscle wasting, menstrual irregularities, gout, postural hypotension, peptic ulcers, and upper gastrointestinal bleeding [92,93]. As expected, hypoglycemic reactions represent the main issues among patients with diabetes, especially in T1DM [92].

Table 2. Studies examining fasting in patients with cardiometabolic risk factors.

Study	Cohort	Fasting Regime	Duration	Results
Wilkinson et al. [31]	19 participants with MetS (13 male; 6 female)	14 h fast (from 8/10 a.m. to 6/8 p.m.)	12 weeks	↓ caloric intake ↓ body weight ↓ body fat and visceral fat ↓ waist circumference ↓ blood pressure ↓ cholesterol (total, LDL)
Parvaresh et al. [91]	69 participants with MetS (35 male; 34 female)	Modified ADF (Sat/Mon/Wed)	8 weeks	↓ body weight ↓ waist circumference ↓ systolic blood pressure ↓ fasting plasma glucose ↔ cholesterol (total, HDL, LDL) ↔ HOMA- IR
Sutton et al. [82]	8 participants (men with prediabetes); proof-of-concept study	eTRF eating period form 8 a.m. to 2 p.m. (while controlling for weight loss)	5 weeks	↓ insulin levels ↓ blood pressure ↓ oxidative stress ↑ insulin sensitivity
Gabel et al. [85]	46 participants with obesity (41 female, 5 male)	16/8 h fasting regime	12 weeks	↓ body weight ↓ energy intake ↓ systolic blood pressure ↔ fat mass ↔ cholesterol ↔ fasting glucose ↔ HOMA IR
Kesztyüs et al. [86]	40 participants with abdominal obesity (31 female, 9 male)	8–9 h TRF	12 weeks	↓ body weight ↓ waist circumference and waist–hip ratio
Chow et al. [89]	20 participants (17 female and 3 male)	16/8 h fasting regime	12 weeks	↓ reduced weight ↓ lean mass ↓ visceral fat ↔ blood pressure ↔ cholesterol ↔ 2 h oral glucose tolerance test
Lowe et al. [84]	116 overweight or obese adults (46 female, 70 male)	TRF (eating from 12–8 pm)	12 weeks	↓ body weight (in TRE group- compared to the starting weight) ↔ body weight between groups

Table 2. *Cont.*

Study	Cohort	Fasting Regime	Duration	Results
Cienfuegos et al. [87]	58 obese adults (53 female, 5 male)	4 h TRF, 6 h TRF	8 weeks	Both regimens achieved: ↓ body weight, ↓ fasting insulin ↓ insulin resistance ↓ oxidative stress
Parr et al. [88]	19 participants with T2DM (10 female, 9 male)	9 h TRF	6 weeks	TRF compliance was achieved ~5 days/week ↔ dietary intake ↔ body weight ↔ glycemic control

Abbreviations: ADF = alternate-day fasting, eTRF = early time-restricted feeding, TRE = time-restricted eating, HOMA-IR = Homeostatic Model Assessment for Insulin Resistance.

7. Conclusions and Future Perspectives

Due to the high MetS prevalence (around 1/3 of the world population), its increasing trend, and high economic burden ($260.6 billion aggregate costs due to obesity in the US), we are in dire need of new dietary, lifestyle, and therapeutic options [3,94]. Diets such as the Mediterranean diet and DASH have proven beneficial, but quality healthy foods such as fruit, vegetables, and fish are more expensive, and hence less accessible to a large part of the population [95,96]. Therefore, diets such as fasting/TRF that do not necessarily need a change in diet quality (with expensive foods) to exert beneficial results could prove valuable in combating this epidemic. A significant amount of evidence was gathered on the efficacy of IF/TRF in animal models [73–76,78]. These studies showed how IF/TRF exerts beneficial effects on the gut microbiota, glucose and insulin metabolism, weight and visceral fat, and lipid metabolism. In other words, fasting affects all the crucial pathophysiological points in MetS/diabetes type II development [4]. However, it is important to acknowledge that different species and the different ages of the tested animals affected the response to IF/TRF, and this variability should therefore be taken into account when assessing metabolic effects of IF/TRF. Considering studies on humans, while the results are promising, they are still scarce (there are only two studies on patients with diagnosed MetS). Most of the studies were performed on overweight/obese individuals who often present at least one more cardiometabolic risk factor, so we can expect similar results in larger studies on MetS patients. It is important to note that the viability and safety of IF/TRF methods have been established in small-scale, pilot studies [31,82,85,89]. The results obtained from human studies are somewhat conflicting, as weight loss was achieved in all studies, whereas in some studies, there was no significant effect on insulin resistance, cholesterol/lipid metabolism, or blood pressure [31,82,84,85,89,91]. However, in some cases, the weight loss was as low as 1–2% of initial body weight. Some of the observed differences might be due to the different genetic makeup of the researched population, differences in the IF/TRF protocols used, and differences in the time of day in which the eating period was set (circadian rhythms).

In conclusion, to establish the significance of fasting in controlling metabolic risk factors and the potential treatment of MetS, there is a need for more randomized control trials on larger cohorts of patients with MetS to gather higher yield evidence. Additionally, economic analysis is required to confirm the presumed cost-utility of IF/TRF.

Author Contributions: J.V., J.B., M.K., and T.T.K. for conceptualization, original draft preparation, and supervision; J.A.B., M.V., D.M., and V.R. for literature review and visualization. All authors contributed to the final draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

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ISBN 978-3-0365-8183-5