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Volume 2 Clinical Evidence

Diet and Metabolic Dysfunction

Edited by
Gaetano Santulli

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Diet and Metabolic Dysfunction

Volume II:
Clinical Evidence

Special Issue Editor

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About the Guest Editor

Gaetano Santulli, M.D., Ph.D. is a principal investigator currently working at Columbia University Medical Center. A physician–scientist, he received his M.D. and Ph.D. at the University of Naples "Federico II". Dr. Santulli completed his training at Columbia University in New York City. Dr. Santulli's expertise comprises both clinical—he is a cardiologist—and basic research topics, including hypertension, diabetes, heart failure, arrhythmias, vascular disease, microRNA, and mitochondrial pathophysiology. He serves on the editorial boards of numerous medical journals. He has authored more than 100 peer-reviewed publications and edited several medical books, including "Adrenal Glands: From Pathophysiology to Clinical Evidence" and "microRNA: From Molecular Biology to Clinical Practice".

Preface to "Diet and Metabolic Dysfunction"

The fundamental importance of diet in the pathophysiology of metabolic syndrome is well acknowledged and may be crucial in the determination of cardiovascular risk and the development of cardiovascular complications. The contributions presented here provide an updated systematic overview examining, in detail, the functional role of different diets and dietary components in maintaining glucose homeostasis and preventing long-term complications. The two books encompass 40 peer-reviewed articles, both in the basic research field (*book 1*) and in the clinical scenario (*book 2*), written by worldwide renowned experts. Intriguingly, one of the assets of the present books is in the melting pot of researchers involved in this project, literally working in all continents, with contributions from United States, Canada, Mexico, Argentina, Italy, Ireland, Spain, Sweden, Austria, Liechtenstein, Germany, Japan, Korea, China, Hong Kong, Taiwan, Malaysia, Saudi Arabia, South-Africa, Nigeria, and Australia. These books include both evidence-based original research and state-of-the-art reviews and meta-analyses of the scientific literature. There are articles investigating different dietary regimens and articles focusing on specific nutrients. In particular, studies on the following topics are presented: omega-3 fatty acids, barley, honey, capsaicin, magnesium, selenium, fructose, vanillic acid, glutamine, histidine, isoleucine and valine, quercetin, rutin, naringin, red ginseng, epigallocatechin gallate (a component of green tea), cudrania tricuspidata fruits, aloe vera, and probiotics and prebiotics. This collection of papers shows that the selection of foods should be based on scientific evidence, knowing the properties of each dietary component.

Gaetano Santulli
Guest Editor

Section 1:
Diet and Cardiovascular Risk

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Review

Nutrition and Cardiovascular Disease: Finding the Perfect Recipe for Cardiovascular Health

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Abstract: The increasing burden of cardiovascular disease (CVD) despite the progress in management entails the need of more effective preventive and curative strategies. As dietary-associated risk is the most important behavioral factor influencing global health, it appears the best target in the challenge against CVD. Although for many years, since the formulation of the *cholesterol hypothesis*, a nutrient-based approach was attempted for CVD prevention and treatment, in recent years a dietary-based approach resulted more effective in reducing cardiovascular risk worldwide. After the publication of randomized trials on the remarkable effects of the Mediterranean diet and the Dietary Approach to Stop Hypertension (DASH) diet on CVD, new efforts were put on research about the effects of complex dietary interventions on CVD. The purpose of this paper is to review the evidence on dietary interventions in the prevention and disease modification of CVD, focusing on coronary artery disease and heart failure, the main disease responsible for the enormous toll taken by CVD worldwide.

Keywords: diet; Mediterranean; DASH; cardiovascular disease; coronary artery disease; heart failure; hypertension

1. Introduction

Even though the global burden of cardiovascular disease (CVD) has steadily decreased during the past 10 years, CVD remains the leading cause of death and disability in developed countries. In fact, CVD is responsible for approximately one of every three deaths in the United States and one of every four deaths in Europe [1,2]. Moreover, developing countries underwent a steep increase in the incidence of CVD over the last 25 years, now being the second cause of years of life lost in most of these countries [3], due in part to their acquisition of Western patterns of diet [4]. The substantial magnitude of the global burden of CVD, despite the progress made in therapy, underscores the need of effective strategies to prevent and modify the course of this widespread disease. Explaining about one-third of global mortality, dietary risk appears to be a priority target for CVD prevention and treatment [3]. The purpose of this paper is to review the evidence on dietary interventions in the prevention and disease modification of CVD, focusing on the most widespread CVD: coronary artery disease (CAD) and heart failure (HF). The authors acknowledge the importance of single nutrients and their role in CVD, however extensive review of their role in CAD and HF is already present in the literature and goes beyond the aim of this paper [5,6].

2. Changing Approaches for Targeting Nutrition in CVD: Still Room for Improvement

The relevant role of nutrition for disease prevention and treatment was already understood in 1747, when James Lind, a Scottish surgeon in the Royal Navy, demonstrated the beneficial effects of citrus fruit for the treatment of scurvy in one of the first clinical trials. The first evidence that nutrition influences the onset and the progression of CVD came in 1908 from the Russian scientist Alexander Ingatowski, who demonstrated that high cholesterol intake caused the development of atherosclerosis in rabbits [7]. Since then, many studies were published that confirmed the role of a fat-enriched diet in the pathogenesis of atherosclerosis, leading to the formulation of the *cholesterol hypothesis* [8]. Following these observations, the first ecologic studies began to develop, such as the pioneering Seven Countries Study, which provided further insights on the impact of different lipids' intake on CVD [9,10]. Moreover, a potential protective role of ω-3 polyunsaturated fatty acids (PUFA) also emerged from observational studies in Eskimos, among which CVD is a rarity [11]. Recently, it was also demonstrated that *n*-3 PUFA exert beneficial effects on endothelial progenitor cell biology [12].

Consequently, in 1957, when the American Heart Association (AHA) Nutrition Committee released the first dietary recommendations, they recognized that "diet may play an important role in the pathogenesis of atherosclerosis and the fat content and the total calories in the diet are probably important factors" [13]. This constituted a milestone of the nutrient-based approach for the prevention and treatment of CVD.

Despite its proven efficacy, this single-nutrient-based strategy appears not to be enough to contrast the onset and the progression of CVD. Indeed, there is growing evidence that, with few exceptions (ω-3 PUFA, sodium, *trans*-saturated fatty acids), single nutrients have effects of limited magnitude on chronic disease, compared with whole foods, or with complex integrated dietary interventions [14]. These and other considerations, such as the difficulties of translating single nutrient-based recommendations into an effective population-wide intervention, led to the advent of a different approach to address nutrition to reduce the burden of CVD, based on foods and dietary patterns rather than on single specific nutrients [15]. These dietary interventions take advantage of the beneficial effects of each of their multiple nutrient components, combining them into healthful diets that achieve greater net effects compared with most single nutrient supplementations. The nutrients and foods act additively and synergistically in the context of each dietary "recipe", though maximizing the magnitude of their final beneficial effects [16].

Despite decades of nutritional research, by 2013 dietary-associated risk was still responsible for 37% of deaths and 24% of disability-adjusted life years (DALYs) for all ages and both sexes [3]. Notably, 9 out of 25 leading global risk factors for DALYs in 2013 were related to inappropriate eating habits (*i.e.*, alcohol use, low intake of fruit, whole grains, vegetables, nuts and seeds, omega-3, fiber, excessive intake of sodium, and iron deficiency) [3]. Effective nutritional interventions, along with promotion of smoke discontinuation and regular practice of aerobic physical activity, are warranted as crucial elements of CVD prevention and regression.

3. Dietary Patterns in Cardiovascular Disease

Despite the extraordinary progress in the treatment of CVD, our knowledge about the cardiovascular effects of diet is still regrettably limited. However, from the 1990s, with the transition from a nutrient-based to a dietary-based approach for addressing nutritional interventions in CVD, new promising data emerged from well-designed randomized trials and meta-analyses. Although the dietary recommendations endorsed by the major Cardiovascular Societies regarding the most widespread CVD, namely CAD and HF, are still based on little firm evidence, unprecedented progress was made in the last few decades in finding novel effective nutritional strategies for CVD prevention and treatment.

3.1. The Mediterranean Diet

In 1970, the American biologist Ancel Keys published the preliminary results of the Seven Countries Study, showing that populations dwelling on the shores of the Mediterranean Sea, in Greece, southern Italy, and the former Yugoslavia, had lower incidence of CAD and CVD in general [9]. Firstly described by Keys himself, the Mediterranean (MED) dietary pattern is rich in whole grains, fruit, vegetables, and low in meat, with a considerable amount of fat deriving from olive oil and nuts (Table 1) [17,18]. This diet seemed to be a possible determinant of the wide difference in CVD prevalence between Mediterranean populations and the Western cohorts in the Seven Countries Study.

Table 1. MED diet and DASH diet composition [19].

MED Diet	DASH Diet
Although there is no uniform definition of the MED diet in randomized trials and cohort studies, the most common features of diets in these studies were the following: -High content in fruits (particularly fresh), vegetables (emphasizing root and green varieties), whole grains (cereals, breads, rice, or pasta), and fatty fish (rich in ω-3 PUFA); -Low content in red meat (emphasizing lean meats); -Substituted lower-fat or fat-free dairy products for higher-fat dairy foods; -Used oils (olive or canola), nuts (walnuts, almonds, or hazelnuts), or margarines blended with rapeseed or flaxseed oils in lieu of butter and other fats.	-High in vegetables, fruits, low-fat fermented dairy products, whole grains, poultry, fish, and nuts; -Low in sweets, sugar-sweetened beverages, and red meats; -Low in saturated fat, total fat, and cholesterol; -Rich in potassium, magnesium, and calcium; -Rich in protein and fiber. **DASH VARIATIONS** In the OMNI-Heart trial, 2 variations of the DASH dietary pattern were compared with DASH: -One that replaced 10% of total daily energy from carbohydrate with protein (mainly non-meat proteins); -Another that replaced the same amount of carbohydrate with unsaturated fat (manly from monounsaturated fatty acids).

DASH, Dietary Approach to Stop Hypertension; MED, Mediterranean, OMNI-Heart, Optimal Macro-Nutrient Intake Heart trial; PUFA, Polyunsaturated Fatty Acids.

3.1.1. Mediterranean Diet and CAD Primary Prevention: From Observational Studies to the PREDIMED Trial

The first pilot studies began analyzing the association between adherence to MED diet and overall survival in the elderly population. In 1995, Trichopoulou and colleagues found out that adherence to MED diet, assessed through a food frequency questionnaire (FFQ) and summarized in a score (a MED score), was strongly associated with overall survival in 187 elderly Greeks. One point increase in the MED score was associated with a 17% increase in overall survival ($p = 0.04$) [20]. This finding was then confirmed in other three prospective cohorts from different geographical regions [21–23]. In 2003, Trichopoulou and colleagues published the results of probably the most important study on MED diet in the primary prevention of CAD and CVD. In a large population-based prospective study involving 22,043 Greeks enrolled in the European Prospective Investigation into Cancer and nutrition (EPIC), with a median follow-up of 44 months, a higher adherence to the MED diet was associated with an increased overall survival. Indeed, a 25% rise in the survival rate was observed every 2 points increase in the MED score assessed at baseline (hazard ratio (HR) 0.75, 95%, confidence interval (CI) 0.64–0.87; $p < 0.001$). The association with the MED score appeared to be evident for mortality from CAD (HR 0.64, 95% CI 0.47–0.94) and, although to a smaller extent, for mortality from cancer (HR 0.76, 95% CI 0.59–0.98), after adjustment for confounding factors [16]. Interestingly, no association emerged between mortality and each of the foods considered in the MED score, thus indicating that the total effect of the whole dietary regimen was stronger than any of the effects of its individual food components [16]. The contribution of the individual components of the MED diet to the overall effect were analyzed by Trichopoulou and her research group after an 8-year follow-up of the Greek cohort of the EPIC. The main contributors to the association of the MED score with mortality were moderate ethanol consumption, low consumption of meat products, high vegetable consumption, and high fruit and nut consumption (Table 2) [24].

Table 2. Components of the MED diet score and their contribution to the association between the MED score and overall mortality in the Greek cohort of the EPIC [21].

Dietary Components of MED Score	Influence on Survival
Ethanol intake (moderate)	24%
Meat and meat products intake (low)	17%
Vegetables intake (high)	16%
Fruits and nuts intake (high)	11%
Monounsaturated:saturated fat ratio (high)	10%
Legumes intake (high)	10%
Dairy products intake (low)	5%
Cereals intake (high)	5%
Fish and seafood (low)	n.s.

EPIC, European Prospective Investigation into Cancer and nutrition; MED, Mediterranean.

These intriguing results were then replicated in larger cohorts worldwide: in 2339 European elderly adults from the Healthy Ageing: a Longitudinal study in Europe (HALE) population [25], in a group of 330,296 US residents enrolled in the National Institutes of Health (NIH)—American Association of Retired Persons (AARP) Diet and Health Study and in a cohort of 74,886 female nurses from the Nurses' Health Study, all of which showed a strong association between adherence to a MED diet and lower all-cause and cause specific (CAD, stroke, CVD or cancer) mortality [26,27]. Noteworthy, the aforementioned studies were epidemiologic prospective studies, comparing cardiovascular outcomes and adherence to the MED diet, assessed through FFQs and expressed as scores. Thus, due to the lack of data from large randomized intervention trials, the evidence supporting the MED diet for the primary prevention of CAD was not enough solid to deserve a strong recommendation by the major Cardiovascular Guidelines. The AHA Guidelines on Lifestyle Management to Reduce Cardiovascular Risk published in 2013 consider the advice to eat a MED diet although judging the level of the supporting evidence as "Low" [19]. Most significantly, the 2012 European Society of Cardiology (ESC) Guidelines on Cardiovascular Disease Prevention recommend following a "healthy diet" rich in fruit, vegetables and fish, but do not mention the MED diet at all [28] (see Table 3 for a summary of the main dietary recommendations for CVD prevention).

Finally, in 2013 stronger evidence supporting the MED diet for the primary prevention of CAD came from the PREvención con DIetaMEDiterránea (PREDIMED) trial. From 2003 to 2006, 7447 Spanish adults with high cardiovascular risk but with no diagnosis of CVD, were randomly assigned in a 1:1:1 ratio to one of three studied diets: a MED diet supplemented with extra-virgin olive oil, a MED diet supplemented with mixed nuts, or a control diet (advice to reduce dietary fat) [29]. Adherence was promoted through quarterly educational sessions and provision of extra-virgin olive oil or mixed nuts, and ensured by regular assessments of self-reported food intake and biomarker analyses. The primary endpoint was the rate of major cardiovascular events (*i.e.*, myocardial infarction, stroke, or cardiovascular death). After a median follow-up of 4.8 years, the primary endpoint occurred in 96 subjects assigned to the MED diet with olive oil (adjusted HR 0.70, 95% CI 0.53–0.91, $p = 0.009$) and 83 subjects assigned to the MED diet with nuts (adjusted HR 0.70, 95% CI 0.53–0.94, $p = 0.02$), *versus* 109 in the control group. Stroke was the most significantly reduced event with the MED diet, followed by myocardial infarction (MI) [29]. Total mortality showed a non-significant trend towards reduction in the MED diet groups, compared with the control group [29]. Although with limitation regarding the possibilities of generalizing its results to non-Mediterranean populations, the 30% reduction of cardiovascular events seen with the MED diet in the PREDIMED trial is truly remarkable and strengthens the evidence in favor of recommending the MED diet for the primary prevention of CAD. Lately, some arguments have been raised about the possible role of lipid intake, from rapeseed oil margarine, olive oil or mixed nuts, in determining the benefits observed in the trials carried out on the MED diet for primary and secondary prevention of CAD [30]. Further randomized trials are needed to confirm or rule out this possibility.

Table 3. ESC and ACC/AHA dietary recommendations for risk factor management and primary prevention of CVD.

Society	Diet Recommendations for CVD—Primary Prevention	COR/LOE
European Society of Cardiology (2012), [28]	A healthy diet is recommended as being the cornerstone of CVD prevention.	I B
	Energy intake should be limited to the amount of energy needed to maintain (or obtain) a healthy weight (BMI < 25 kg/m^2).	-
	Saturated fatty acids to account for <10% of total energy intake, through replacement by PUFA.	-
	Trans unsaturated fatty acids <1% of total energy intake.	-
	<5 g of salt per day.	-
	30–45 g of fiber per day, from wholegrain products, fruits and vegetables.	-
	200 g of fruit per day (2–3 servings).	-
	200 g of vegetables per day (2–3 servings)	-
	Fish at least twice a week, one being oily fish.	-
	Consumption of alcoholic beverages should be limited to 2 glasses per day (20 g/day of alcohol) for men and 1 glass per day (10 g/day of alcohol) for non-pregnant women.	-
	In general, when following the rules for a healthy diet, no dietary supplements are needed.	-
American College of Cardiology/American Heart Association (2013), [19]	**LDL-C: Advise adults who would benefit from LDL-C lowering to:**	
	1. Consume a dietary pattern that emphasizes intake of vegetables, fruits, and whole grains; includes low-fat dairy products, poultry, fish, legumes, non-tropical vegetable oils, and nuts; and limits intake of sweets, sugar-sweetened beverages, and red meats. a. Adapt this dietary pattern to appropriate calorie requirements, personal and cultural food preferences, and nutrition therapy for other medical conditions (including diabetes). b. Achieve this pattern by following plans such as the DASH dietary pattern, the USDA Food Pattern, or the AHA Diet.	I A
	2. Aim for a dietary pattern that achieves 5%–6% of calories from saturated fat.	I A
	3. Reduce percent of calories from saturated fat	I A
	4. Reduce percent of calories from *trans* fat.	I A
	BP: Advise adults who would benefit from BP lowering to:	
	1. Consume a dietary pattern that emphasizes intake of vegetables, fruits, and whole grains; includes low-fat dairy products, poultry, fish, legumes, non-tropical vegetable oils, and nuts; and limits intake of sweets, sugar-sweetened beverages, and red meats. a. Adapt this dietary pattern to appropriate calorie requirements, personal and cultural food preferences, and nutrition therapy for other medical conditions (including diabetes). b. Achieve this pattern by following plans such as the DASH dietary pattern, the USDA Food Pattern, or the AHA Diet.	I A
	2. Lower sodium intake.	I A
	3. Specifically: a. Consume no more than 2400 mg of sodium/day; b. Further reduction of sodium intake to 1500 mg/day can result in even greater reduction in BP; and c. Even without achieving these goals, reducing sodium intake by at least 1000 mg/day lowers BP.	IIa B
	4. Combine the DASH dietary pattern with lower sodium intake.	I A

ACC, American College of Cardiology; AHA, American Heart Association; BMI, body mass index; COR, class of recommendation (I: recommended/indicated; IIa: should be considered); CVD, cardiovascular disease; DASH, dietary approach to Stop Hypertension; ESC, European Society of Cardiology; LDL-C, low density lipoprotein cholesterol; LOE, level of evidence (A: data derived from multiple randomized clinical trials or meta-analyses; B: data derived from a single randomized clinical trial or large non-randomized studies); PUFA, Polyunsaturated Fatty Acids; USDA, United States Department of Agriculture.

3.1.2. Mediterranean Diet and CAD Secondary Prevention: From Lyon Heart Study to Present Days

The Lyon Heart Study was a landmark study of the MED diet tested for the secondary prevention of CAD [31,32]. From 1988 to 1992, 605 survivors after a first MI were enrolled and randomized either to a control group, receiving dietary advice for a "prudent" low-fat diet, or the experimental group, undergoing an hour-long educational session about the MED diet and supplied a rapeseed oil based margarine comparable in composition to olive oil, but more palatable to the study population. Moderate alcohol consumption was allowed. Although serum lipids, body mass index (BMI) and blood pressure (BP) remained similar in the two groups, after a mean follow up of 27 months only three cardiac deaths occurred in the experimental group *versus* 16 in the control group (relative risk (RR) 0.27, 95% CI 0.12–0.59, $p = 0.001$), and overall mortality was eight subjects in the experimental group *versus* 20 in the control group (RR 0.30, 95% CI 0.11–0.82, $p = 0.02$) [31]. By the end of the study, after a mean follow-up of 46 months all the composite outcomes, combining cardiac death and non-fatal MI with other events, were significantly reduced in the MED group [32]. Although no other randomized trial was carried out for secondary prevention of CAD with the MED diet, prospective studies published in the last 15 years confirmed the findings of the Lyon Heart Study [33,34]. Intriguingly, one randomized trial comparing dietary intervention (101 patients, 50 randomized to a low-fat diet and 51 to the MED diet) *versus* usual post-MI care, found that dietary intervention *per se* was beneficial after MI [35]. The relatively short time spent for dietary advice and the lenient follow-up schedule in the Lyon Heart Study suggest that benefits from dietary interventions on CVD are achievable with limited effort and are feasible on large-scale. Secondly, dietary intervention showed a complementary beneficial role beside pharmacological treatment in post-MI care. Currently, the MED diet appears to be the only dietary pattern supported by a large randomized trial for the secondary prevention in patients with established CAD [36,37] (See Table 4 for a summary of the main dietary recommendations for CAD secondary prevention).

Table 4. ESC and ACC/AHA dietary recommendations for secondary prevention of CAD.

Society	Diet Recommendations for CAD—Secondary Prevention	LOE
European Society of Cardiology (2013), [36]	Energy intake should be limited to the amount of energy needed to maintain (or obtain) a healthy weight (BMI < 25 kg/m^2).	-
	Saturated fatty acids to account for <10% of total energy intake, through replacement by PUFA.	-
	Trans unsaturated fatty acids <1% of total energy intake.	-
	<5 g of salt per day.	-
	30–45 g of fiber per day, from wholegrain products, fruits and vegetables.	-
	200 g of fruit per day (2–3 servings).	-
	200 g of vegetables per day (2–3 servings)	-
	Fish at least twice a week, one being oily fish.	-
	Consumption of alcoholic beverages should be limited to 2 glasses per day (20 g/day of alcohol) for men and 1 glass per day (10 g/day of alcohol) for non-pregnant women.	-

Table 4. *Cont.*

Society	Diet Recommendations for CAD—Secondary Prevention	LOE
American College of Cardiology/American Heart Association (2012), [37]	Dietary therapy for all patients should include reduced intake of saturated fats (to <7% of total calories), *trans* fatty acids (to <1% of total calories), and cholesterol (to <200 mg/day)	B
	All patients should be counseled about the need for lifestyle modification: weight control; increased physical activity; alcohol moderation; sodium reduction; and emphasis on increased consumption of fresh fruits, vegetables, and low-fat dairy products	B
	BMI and/or waist circumference should be assessed at every visit, and the clinician should consistently encourage weight maintenance or reduction through an appropriate balance of lifestyle physical activity, structured exercise, caloric intake, and formal behavioral programs when indicated to maintain or achieve a BMI between 18.5 and 24.9 kg/m^2 and a waist circumference less than 102 cm (40 inches) in men and less than 88 cm (35 inches) in women (less for certain racial groups)	B
	In patients with symptomatic ischemic heart disease who use alcohol, it might be reasonable for non-pregnant women to have 1 drink (4 ounces of wine, 12 ounces of beer, or 1 ounce of spirits) a day and for men to have 1 or 2 drinks a day, unless alcohol is contraindicated (such as in patients with a history of alcohol abuse or dependence or with liver disease).	C

ACC, American College of Cardiology; AHA, American Heart Association; BMI, body mass index; CAD, coronary artery disease; ESC, European Society of Cardiology; LOE, level of evidence (B: data derived from a single randomized clinical trial or large non-randomized studies; C: consensus of opinion of the experts and/or small studies, retrospective studies, registries); PUFA, Polyunsaturated Fatty Acids.

3.1.3. Mediterranean Diet and Heart Failure

Heart failure is a CVD characterized by a severe prognosis. Moreover, patients with heart failure often have comorbidities that negatively affect the prognosis, including kidney disease, anemia, respiratory disorders and depression [38–40]. Chronic heart failure progression is modifiable using therapies that antagonize adverse neuro-hormonal pathways (beta-blockers, angiotensin-converting-enzyme inhibitors, angiotensin receptor blockers, and mineralocorticoid antagonists) while diuretics are effective in treating congestion and HF symptoms [41,42]. Despite the current improvement in the management of chronic heart failure, none of the available treatments has demonstrated to improve in prognosis in acute heart failure, except a new vasodilator whose trial is still underway [43,44].

Beside the above mentioned therapies, there is actually a solid rationale for the beneficial effects of nutritional interventions on HF, especially regarding the MED diet (see Table 5 for a summary of the main dietary recommendations for HF) [45,46].

Notably, multiple risk factors (e.g., hypertension, diabetes) as well as pathophysiological mechanisms (e.g., systemic inflammation, neurohormonal activation) may be positively influenced by the MED diet [47,48].

A meta-analysis by Nordmann and colleagues, showed significant benefits of the MED diet, compared with low-fat diets, in reducing BMI, systolic blood pressure (SBP), fasting plasma glucose, total cholesterol, and high-sensitivity C-reactive protein [49]. Moreover, MED diet adherence was associated with lower serum concentrations of biomarkers related to inflammation and endothelial dysfunction, in a cohort from the Nurses' Health Study [50], and with lower serum lipids and oxidized LDL, in a randomized sample from the PREDIMED trial [51]. It is therefore in line with these findings, that data collected from prospective cohorts showed an association between adherence to the MED diet and lower incidence of HF both in men (multivariable RR for the highest *vs.* lowest quartile of MED score 0.69, 95% CI 0.57–0.83) and in women (RR 0.79, 95% CI 0.68–0.93, $p = 0.004$) [52,53].

Table 5. ESC and ACC/AHA dietary recommendations for HF.

Society	Diet Recommendations for HF	COR/LOE
European Society of Cardiology 2012 [45]	An ω-3 PUFA preparation may be considered to reduce the risk of death and the risk of cardiovascular hospitalization in patients treated with an angiotensin converting enzyme inhibitor (or angiotensin receptor blocker), beta-blocker, and an mineral corticoid receptor antagonist (or angiotensin receptor blocker).	IIb B
	Avoid excessive fluid intake: fluid restriction of 1.5–2 L/day may be considered in patients with severe HF to relieve symptoms and congestion. Restriction of hypotonic fluids may improve hyponatremia. Routine fluid restriction in all patients with mild to moderate symptoms is probably not of benefit. Weight-based fluid restriction (30 mL/kg body weight, 35 mL/kg if body weight >85 kg) may cause less thirst	-
	Monitor and prevent malnutrition.	-
	Eat healthily and keep a healthy weight.	-
	Modest intake of alcohol: abstinence is recommended in patients with alcohol-induced cardiomyopathy. Otherwise, normal alcohol guidelines apply (2 units per day in men or 1 unit per day in women). Note: 1 unit is 10 mL of pure alcohol (e.g., 1 glass of wine, 1/2 pint of beer, 1 measure of spirit).	-
	Sodium restriction may help control the symptoms and signs of congestion in patients with symptomatic HF classes III and IV.	-
American College of Cardiology/American Heart Association (2013) [4]	STAGE A: hypertension and lipid disorders should be controlled in accordance with contemporary guidelines to lower the risk of HF.	I A
	STAGE B: in patients with structural cardiac abnormalities, including left ventricular hypertrophy, in the absence of a history of MI or acute coronary syndrome, BP should be controlled in accordance with clinical practice guidelines for hypertension to prevent symptomatic HF	I A
	STAGE C: sodium restriction is reasonable for patients with symptomatic HF to reduce congestive symptoms.	IIa C
	STAGE C: ω-3 PUFA supplementation is reasonable to use as adjunctive therapy in patients with NYHA class II–IV symptoms and HFrEF or HFpEF, unless contraindicated, to reduce mortality and cardiovascular hospitalizations.	IIa B
	STAGE C: nutritional supplements as treatment for HF are not recommended in patients with current or prior symptoms of HFrEF.	III B
	STAGE C: Routine use of nutritional supplements is not recommended for patients with HFpEF.	III C
	STAGE D: fluid restriction (1.5 to 2 L/day) is reasonable in stage D, especially in patients with hyponatremia, to reduce congestive symptoms.	IIa C

ACC, American College of Cardiology; AHA, American Heart Association; BP, blood pressure; COR, class of recommendation (I: recommended/indicated; IIa: should be considered; IIb may be considered; III: not recommended); ESC, European Society of Cardiology; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LOE, level of evidence (A: data derived from multiple randomized clinical trials or meta-analyses; B: data derived from a single randomized clinical trial or large non-randomized studies; C: consensus of opinion of the experts and/or small studies, retrospective studies, registries); MI, myocardial infarction; NYHA, New York Heart Association; PUFA, polyunsaturated fatty acids.

Given these results, it is therefore not surprising that MED diet was shown to influence also HF progression and mortality. An interesting association of the MED diet with an improvement of ventricular function was suggested by a study conducted by Chrysohoou and colleagues, demonstrating that high adherence to the MED diet was associated with improvement in left ventricular ejection fraction (LVEF) and diastolic function [54,55]. Additionally, data from the PREDIMED study showed a reduction in serum biomarkers related with HF in the groups randomized to the MED diet (mean NT-proBNP reduction: −84.7 pg/mL, 95%CI −145 to −24.5, $p = 0.006$) [56]. Other prospective studies provided insights on the favorable association of MED diet with lower incidence of sudden cardiac death (SCD), one of the main causes of death in patients with HF. In a study by Bertoia *et al.* on 93,000 women enrolled in the Women's Health Initiative, MED diet, but not DASH diet adherence, was associated with lower risk of SCD (highest to lowest quintile HR 0.64, 95% CI 0.43–0.94) [57], a result emphasized also in a cohort from the Nurses' Health Study [58]. Finally, a considerable amount

of studies showed interesting associations of MED diet adherence with lower incidence of various conditions that are usually present as comorbid diseases in HF patients [59]. The results of multiple prospective trials conducted until 2013 are well summarized in a meta-analysis by Sofi and colleagues showing substantial benefit of the MED diet on overall, cardiovascular and non-cardiovascular mortality [60]. A consistent number of studies, published during the past decade, further confirmed the association of MED diet adherence and favorable outcome of various HF comorbidities, like diabetes, metabolic disease and obstructive lung disease [61,62].

All those data may explain the reduced risk of death observed in HF patients following a MED diet pattern. This reduced risk of death was demonstrated in a population of 37,308 men from the Cohort of Swedish Men (RR of HF mortality: 0.55; 95% CI 0.31–0.98) [52], and observed as a non-significant trend among 3215 female participants of the Women's Health Initiative (RR highest to lowest quartile: 0.85; 95% CI, 0.70–1.02) [63]. Although there is need of more conclusive data from randomized intervention trials, these results provide encouraging evidence on the benefits of a MED-style diet for HF patients.

The pathophysiological mechanisms that may explain these beneficial effects of diet in HF are not limited to those previously mentioned. In addition, several micronutrient deficiencies (*i.e.*, iron, coenzyme Q10, vitamin D, thiamine, and amino acids) have been described in HF [64,65] that may benefit from dietary supplementation, although specific studies led to controversial results [64,66,67]. Likely, an integrated dietary intervention, with a well-balanced food composition along with a comprehensive micronutrient content could be the most successful nutritional strategy in HF.

3.1.4. A Cluster of Definitions: What Does *Mediterranean Diet* Mean Today?

Given the social and cultural changes in alimentary habits and tastes from 1960s to present days, the current MED diet is not the same as the one that Cretan people ate at the time of the Seven Countries Study. As a consequence various studies, carried out in different decades, used different FFQ and MED scores. A brief description of the current MED diet based on the most significant studies and trials that demonstrated its properties has been provided by the AHA in the Guidelines on Lifestyle Management to Reduce Cardiovascular Risk and is summarized in Table 1 [19]. Recently, a new MED diet pyramid based on scientific evidence and epidemiological studies was elaborated to summarize the MED dietary pattern as it can be applied to present days and adapted to different geographical, cultural and socio-economic contexts [68].

3.2. The DASH Diet

In the 1990s, the prevalence of hypertension, one of the main determinants of CVD, had already reached the proportions of an epidemic among the American population [69]. Following the observation that vegetarians tended to have lower BP values than non-vegetarians [70], a Collaborative Research Group led by Lawrence Appel tested the effects of a diet rich in fruit, vegetables and low-fat dairy foods on blood pressure in a multicenter randomized feeding study: the Dietary Approach to Stop Hypertension (DASH) trial. This trial enrolled 459 adults with SBP lower than 160 mmHg and diastolic blood pressure (DBP) of 80 to 95 mmHg, not on BP-lowering medications. Participants were randomly assigned to eight weeks feeding with a control diet, similar in composition to the average American diet, or a diet rich in fruit and vegetables, or a "combination" diet (hereafter referred to as the DASH diet) rich in fruit, vegetables, and low-fat dairy products with a reduced content of saturated and total fat (components of the DASH diet are listed in Table 1). After 8 weeks, compared with the control diet, the fruits-and-vegetables diet reduced SBP by 2.8 mmHg ($p < 0.001$) and DBP by 1.1 mmHg ($p = 0.007$), and the combination diet reduced SBP by 5.5 mmHg and DBP by 3 mmHg ($p < 0.001$ each) [71]. The effects of the combination diet were even more pronounced among the 133 subjects with hypertension, which experienced a mean reduction in SBP of 11.4 mmHg and in DBP of 5.5 mmHg compared with the controls ($p < 0.001$ each) [71]. The DASH trial presented a strong study design leading to minimization of potential biases. A significant strength of the study was the

fact that all meals were prepared in the research kitchen, enabling full control of the food and nutrient composition of the studied diets.

Further analyses showed that the DASH diet reduced total (−13.7 mg/dL) and LDL (−10.7 mg/dL) cholesterol (all $p < 0.0001$) [72]. A subgroup analysis demonstrated a greater effect on SBP in African Americans (−6.8 mmHg) than in whites (−3.0 mmHg) ($p < 0.05$) [73]. In addition, data from one-year follow-up of the study population, showed sustained reductions of BP and a positive influence over time on eating habits in DASH diet group [74]. Subsequent studies proved that the DASH diet does not exert a simple "cosmetic" effect on BP values, but it is also contrasts inflammation and the detrimental effects of hypertension on organ damage. In 2009, Jacobs and colleagues showed that the DASH dietary pattern led to a reduction of albumin excretion rate (AER) [75]. This finding was confirmed in the CARDIA study, that demonstrated an association between scarce adherence to the DASH dietary pattern and obesity with incident microalbuminuria in a young healthy population [76].

3.2.1. Unity Makes Strength: The DASH-Sodium Trial

The DASH-sodium trial was designed to assess whether a low-sodium content could improve the benefits of the DASH diet alone [77]. Participants were assigned to a diet, either DASH or control, each combined with high (*i.e.*, 150 mmol/day of sodium, reflecting typical consumption in the US), or intermediate (*i.e.*, 100 mmol/day, corresponding to the upper limit of the National Recommendations in 1997), or low sodium content (*i.e.*, 50 mmol/day, a level hypothesized to produce an additional lowering in BP). In this trial, the reduction of sodium intake produced an additional significant BP lowering effect both combined with the DASH diet (3 mmHg from high to low sodium level, $p < 0.01$), or the control diet (6.7 mmHg from high to low sodium level, $p < 0.001$). The BP lowering effect of reduced sodium intake was observed in all the analyzed subgroups, though more pronounced among black, hypertensive and female subjects [78].

3.2.2. Theme and Variations: The OMNI-Heart Trial

After the encouraging results of the previous DASH trials, the Optimal Macro-Nutrient Intake Heart trial (OMNI-Heart) was carried out to test the potential benefits of a DASH diet with varying content in macronutrients, on CVD risk [79]. A sample of 164 pre-hypertensive or mild hypertensive subjects with LDL cholesterol (LDL-C) <220 mg/dL, triglycerides (TG) <750 mg/dL, not on medications influencing BP or blood lipids, was randomized to a sequence of three diets. One of the diets was a carbohydrate-rich diet similar to the DASH diet, the other two were modified DASH diets: a protein-rich diet, and an unsaturated fatty acids-rich diet (see Table 1). All three dietary patterns were produced significant lowering of BP and LDL-C values from baseline, with greater results with the protein- and unsaturated fatty acids-rich diets [80].

3.2.3. DASH Diet from Risk Factors Reduction to CAD Prevention

The aforementioned DASH diet trials showed remarkable effects on BP and lipid profile that encompass all degrees of hypertension, making it a useful tool for population-wide interventions aimed at reducing cardiovascular risk. Notably, the effects of the DASH diet were more pronounced among hypertensive subjects compared with the normotensive, thus making it an even more appropriate strategy for initial treatment of hypertension [71]. Moreover, subsequent studies demonstrated that the DASH diet led to further BP decrease compared with the pharmacological therapy alone, when added to either losartan or candesartan [81,82], thus extending its benefits also in patients on BP-lowering drugs. Finally, the more pronounced effects of the DASH diet among black patients, make it an useful tool to reduce cardiovascular risk in developing countries, whose results have already been encouraging in some pilot studies [83].

Besides risk factor reduction, the DASH diet has also other potential benefits in the setting of CAD prevention. In a prospective cohort of 88,517 females from the Nurses' Health Study, Fung and colleagues found that a high DASH adherence score was associated with less inflammation (assessed

as C-reactive protein and interleukin-6 serum concentration) [84]. Interestingly, in a cross-sectional study on 148 adults undergoing coronary angiography, greater adherence to the DASH diet was associated with lower concentrations of asymmetrical dimethyl-arginine, a marker of endothelial dysfunction, which was associated with the presence of CAD [85]. Moreover, in 2008, Fung and colleagues demonstrated that adherence to a DASH diet, assessed seven times during 24 years of follow-up in a prospective cohort of 88,517 female nurses without prior history of CVD, was associated with significantly lower risk of CAD (RR across quintiles 1.0, 0.99, 0.86, 0.87, and 0.76, $p < 0.001$ for trend) and stroke (RR across quintiles 1.0, 0.92, 0.91, 0.89, and 0.82; $p = 0.002$ for trend) [84]. Thus, given the strength of the DASH trial findings, supported also by subsequent studies showing their successfully reproducibility in clinical practice [86], in 2013 the AHA Guidelines on lifestyle management to reduce cardiovascular risk recommended the DASH diet with "strong" level of evidence (LOE) to reduce cardiovascular risk [19]. Most importantly, the DASH diet was also associated with increased survival. In 2009, Parikh and colleagues found an association with DASH diet adherence and lower all-cause mortality among 5,532 hypertensive adults in the Third National Health and Nutrition Examination Survey (HR 0.69, 95% CI 0.52–0.92; $p = 0.01$) [87].

The above mentioned studies confirm the beneficial effects of this favorable dietary pattern not only on BP values, but also on inflammation and on the micro- and macrovascular damage, multiple additive beneficial effects that efficiently sum up to achieve the final goal of reducing cardiovascular events. Although randomized trials are needed to establish whether the DASH diet could be beneficial in the primary and secondary prevention of CAD there is a strong rationale supporting its potential beneficial effects also in that context (see Tables 3 and 4 for a summary of the main dietary recommendations for CAD prevention).

3.2.4. DASH Diet and Heart Failure

The DASH diet exerts positive effects also in patients with HF. Recently, Levitan and colleagues demonstrated that high adherence to a DASH diet was associated with lower incidence of HF, compared with low DASH diet adherence, in a prospective cohort of women from the Swedish Mammography Cohort (RR highest to lowest quartile 0.63, 95% CI 0.48–0.81; p for trend < 0.001) [88], and with lower HF deaths and hospitalizations in a prospective cohort of men from the Cohort of Swedish Men (22% lower rate of HF events in the highest *vs.* lowest quartile, 95% CI 5%–35%, p for trend = 0.006) [89]. These results have been confirmed by a subsequent meta-analysis displaying that following the DASH diet can significantly protect against the most widespread CVD, reducing the risk of CVD, CHD, stroke, and HF by 20%, 21%, 19% and 29%, respectively (all $p < 0.001$) [90]. Finally, an analysis by Levitan *et al.* in a prospective cohort from the Women's Health Study observed a relative risk reduction of HF mortality of 16% across quartiles of the DASH diet score (RR of HF mortality 0.84, 95% CI 0.70–1.00; p for trend = 0.01) [63].

In the past decade, some small studies have explored the mechanisms leading to this reduction in HF incidence, progression and mortality. First, as previously discussed, hypertension is one of the main contributors to the pathogenesis of HF, especially HF with preserved ejection fraction (HFpEF) [91]. In a small study of 13 patients with hypertension and stable HFpEF, a sodium restricted DASH diet resulted in significant reduction of BP, along with a reduction in carotid-femoral pulse wave velocity, an index of arterial stiffness [92]. A DASH dietary pattern was also associated with improvements in left ventricular diastolic function, arterial elastance, and ventricular-arterial coupling in patients with HFpEF [93]. Finally, using targeted metabolomics to explore metabolite changes, proof was provided that a sodium restricted DASH could improve myocardial energy substrate utilization in patients with HFpEF [94]. The DASH diet was also demonstrated to be positively associated with left ventricular contractile function, thus providing potential benefits to patients suffering of HF with reduced ejection fraction (HFrEF). In the Multi-Ethnic Study of Atherosclerosis (MESA), a 1-unit increase in DASH diet score was significantly associated with an increase in stroke volume (+0.10 mL/m^2), with a non-significant trend towards an increase in left ventricular ejection fraction (+0.04%, $p = 0.08$) [95].

In addition, the DASH diet improved symptoms and quality of life (QoL) in patients with established symptomatic HF. In a small randomized trial of 48 stage C chronic HF patients, an improvement in exercise capacity (292 m *vs*. 197 m; $p = 0.018$) and QoL scores (21 *vs*. 39; $p = 0.006$) were observed in patients randomized to the DASH diet [96]. Finally, early studies provided insights about a possible advantageous effect of this diet in relieving congestion often associated with HF. In 2003 Akita *et al*. performed an analysis of the BP-natriuresis relationship in patients enrolled in the DASH-sodium trial. The results showed that the DASH diet had the effect of steepening the x-y relationship between BP and natriuresis (slope was increased from 29.5 ± 3.4 to 64.9 ± 13.1 mmol/day/mmHg, $p = 0.0002$), providing the first evidence of a possible natriuretic effect of the DASH diet [97].

After the publication of these encouraging data, randomized trials are needed to get more conclusive data on the beneficial effects of the DASH diet in patients with preclinical or established HF, thus enabling the formulation of more specific guidelines to deal with the complex problem of nutrition in patients with HF (see Table 5 for a summary of the main dietary recommendations for HF).

3.3. The Next Future: Promising Dietary Patterns

Beside the above-mentioned dietary patterns, some other approaches have been described and tested that may represent valid tools for CVD prevention and treatment. They are either empirically derived dietary patterns, or hypothesis driven dietary patterns [5]. The former are dietary patterns observed to be beneficial on CVD in epidemiological studies, which have been subsequently analyzed and then tested in prospective or interventional studies. The latter are either based on diet quality or on adherence to dietary guidelines or are groups of food expected to act synergistically on a common target, and thus are artificial dietary models. In the next future, these diets will be possibly tested on hard endpoints in large randomized trials, hopefully confirming the encouraging results of the early studies.

3.3.1. Empirically Derived Dietary Patterns

The Japanese Diet. Following the observation that Japanese inhabitants of the Okinawa Prefecture have the longest life expectancy in Japan and likely in the world [98], it has been hypothesized that their traditional diet rich in fish, seaweed, soybean products, vegetables and green tea, may convey health benefits. Although the single components of this diet have been associated with cardiovascular benefits [99–101] only few studies investigated the effects of the whole Japanese dietary pattern on CVD. Interestingly, despite being associated with higher prevalence of hypertension, probably due to its high sodium intake, the traditional Japanese dietary pattern, after adjustment for potential confounders, showed to reduce the risk of CVD mortality [102]. On the other hand, a recent study by Niu *et al*. showed that a traditional Japanese diet was associated with lower BP, although the sodium content of the diet pattern followed by the study participants was not specified [103]. Taken together, these results show that the Japanese dietary patter seem to exert favorable effects on CVD regardless of its effects on BP. These preliminary results deserve further research to better characterize the benefits of this dietary pattern while providing new insights on the role of BP on CVD.

The Nordic Diet. During the last five years, the effects of a Nordic Diet (ND) including oily fish (salmon and mackerel), vegetables, roots, legumes, fruits, berries and wholegrain cereals (oat, rye, and barley) [104], were studied in epidemiological studies and randomized trials. The first randomized trial on ND, carried out in 2007–2008, showed a significant reduction in total cholesterol (-0.98 ± 0.75 mmol/L, $p < 0.0001$) and LDL-C (-0.83 ± 0.67 mmol/L, $p < 0.001$) as well as in weight (-3 ± 1.86 Kg, $p < 0.001$) and SBP (-6.55 ± 13.18 mmHg, $p = 0.008$) in six weeks, among those randomized to ND compared with controls [105]. Following this trial, other feeding trials confirmed the effects of the ND on hypertension and blood lipids, and showed that ND exerts positive effects also on inflammation, insulin sensitivity and body weight [106,107]. Adherence to the ND, assessed with FFQ, was even associated with lower risk of all-cause mortality in two large cohort studies [108,109]. However, a recent cohort study on a large sample of Swedish women did not find a significant

association between adherence to a ND and a reduction of risk of CVD [110]. Even though a lack of accuracy in the ND adherence score has been proposed to explain this surprising result [111], new prospective studies and trials are warranted to clarify the effects of this dietary pattern.

The Vegetarian Diet. The association between a vegetarian diet and lower BP values has been known since the 1970s, and was actually confirmed by the lower BP values observed in the vegetable-rich-diet arm of the DASH trial [70,71]. A recent meta-analysis observed that a vegetarian diet significantly lowered blood cholesterol levels, LDL-C, HDL-C, and non-HDL-C, without affecting TG [112]. In another meta-analysis, vegetarian diet reduced significantly the risk of incidence and/or mortality from ischemic heart disease (RR 0.75; 95% CI, 0.68–0.82) [113]. However, other single studies and pooled analysis failed to confirm these results [114,115]. Similar to the Japanese diet, these results underscore the concept that BP cannot be linked *a priori* to cardiovascular outcomes, and that further prospective and randomized trials are needed.

3.3.2. Hypothesis Driven Dietary Patterns

The Portfolio Diet. The "portfolio" diet is a dietary approach meant to achieve effective cholesterol reduction through a combination diet of functional foods or foods containing specific therapeutic components. The basic idea, first proposed in 1999, was to combine into one diet viscous fiber, soy, almonds, plant sterols and stanols [116]. Various studies tested different food combinations obtaining remarkable reductions in LDL-C ranging from 4% to 35% [117]. Noteworthy, in a randomized study, Jenkins and colleagues assigned 48 subjects in a 1:1:1 ratio to a diet very low in saturated fat (control), or the same diet plus lovastatin 20 mg (statin), or the portfolio diet. After one month, the control, statin, and dietary portfolio groups showed mean decreases in LDL-C of 8.0% $\pm$ 2.1% ($p = 0.002$), 30.9% $\pm$ 3.6% ($p < 0.001$), and 28.6% $\pm$ 3.2% ($p < 0.001$), respectively, thus demonstrating that the efficacy of this dietary portfolio was comparable to a statin therapy [118]. Further studies demonstrated that dietary portfolio reached a significant BP-lowering effect, comparable to that of the DASH diet [119], and a positive effective risk factor management in patients with established CAD [120].

The Glycemic Index Diet. In 1981, Jenkins and colleagues first published a paper on the effects of various food on blood glucose levels [121]. The authors concluded that a diet based on GI might be particularly promising for nutrition in diabetics or in patients with a metabolic disease [121]. During the following years a dietary approach based on GI or glycemic load (GL, *i.e.*, the GI of a food multiplied by its carbohydrate content) was tested on various conditions, and also in CVD. Some meta-analysis reported that GI was associated with significant increased risk of CVD [122,123], but other studies led to conflicting results [124,125]. Interestingly, a six-month randomized controlled trial conducted in 122 overweight and obese adults (the GLYNDIET study), showed that a low-GI and energy-restricted diet may be more effective than a high-GI and low-fat diet at reducing body weight and controlling glucose and insulin metabolism [126]. These results suggest that a GI-based diet may be more useful in diabetic patients than in non-diabetics. Further studies are needed to clarify the effects of GI-based diets on CVD according to gender, weight and concomitant metabolic diseases.

4. Beyond Nutrients: Which Is the Optimal Amount of Salt to Flavor a Healthful Diet?

Current guidelines recommend to reduce sodium intake either for primary or secondary prevention of CAD and CVD (Tables 3 and 4). The recommended targets vary from 4 to 5 g of salt per day, corresponding to 1550–2000 mg of sodium. When considering HF, some guidelines even recommend salt restriction to 1–2 g per day, in case of advanced symptoms [127]. In fact, the DASH-sodium trial [78] and large randomized trials, such as the INTERSALT and INTERMAP [128,129], provided evidence of the beneficial effects of a low sodium diet for hypertension, but should sodium restriction be recommended also to non-hypertensive patients? While some authors have claimed that a salt-restricted diet could reduce cardiovascular risk [130,131], others trials and meta-analyses supported the opposite viewpoint [132,133].

In 2013, the National Institute of Medicine reviewed the existing evidence for sodium restriction in CVD and concluded that there was not sufficient evidence from solid studies to support the recommendation of sodium restriction to prevent and treat CVD, except from hypertension. Actually recent studies in HF setting provided evidence that sodium restriction may even worsen clinical outcomes. In a prospective study of 244 patients with HF, Song and colleagues demonstrated that patients in NYHA class I/II with <2 g/day sodium intake had a 3.7-times higher risk ($p = 0.025$) for hospitalization or death than those with 2–3 g/day sodium intake after controlling for covariates. Conversely, in NYHA class III/IV, >3 g/day sodium intake predicted shorter event-free survival ($p = 0.044$), whereas there was no difference in survival curves between patients with <2 g/day and those with 2–3 g/day sodium intake [134]. Recently, a study by Doukky *et al.* has been published that suggests that sodium restriction may increase hospitalizations in patients with NYHA II/III HF, especially in patients not receiving angiotensin-converting enzyme inhibitor or angiotensin receptor blocker (HR: 5.78; 95% CI: 1.93 to 17.27; $p = 0.002$), thus suggesting that sodium intake produces not only hemodynamic but also neurohormonal changes [135].

The publication of the document by the National Institute of Medicine and of the subsequent studies, gave the beginning to a new wave of research on potential benefits and drawbacks of sodium intake in CVD. Some randomized trials are currently ongoing to determine the actual effects of salt in different cardiovascular diseases [136].

5. Dietary Interventions and the Real World: The Complex Issue of Translating Knowledge into Practice

Since their publication, the dietary patterns supported by the strongest evidence, namely the MED and the DASH, have been recommended by most Cardiovascular Societies worldwide. However, the adherence of general population to these dietary patterns is very low and with a temporal trend towards divergence from these diet models, especially in the subgroups expected to receive greater benefits from these diets, thus neutralizing the potential benefits of these "weapons" [137].

There are several reasons that may explain poor adherence to a virtuous dietary pattern. In first place, despite health promotion programs, secular trends and food industry induce an increase in consumption of highly-refined energy-dense products instead of low-fat, fresh food [138]. Secondly, recommended diet patterns are, at various extents, more costly than the average Western food patterns [139–141]. For instance, DASH diet costs $130 per week for a family of four, having been classified in a "low" to "moderate" cost category according to the USDA estimates [73]. Notably, a recent study of 2181 Spanish subjects, found out that every 1€ increase of the diet cost per 8.36 MJ was associated with an average 300 hg decrease in body weight and a 0.1 Kg/m^2 decrease in BMI ($p = 0.02$ and $p = 0.04$, respectively), thus confirming that improvements in diet quality entail increases in diet costs [142]. Thirdly, fresh produce and groceries appear to be less available in urban communities, where most of the high-risk population dwells [143]. In fourth place, the lack of palatability of this kind of diet has been proved a further reason of scarce adherence to a healthful kind of diet, especially among African Americans [144]. Finally, education, social status and the presence of other lifestyle- and behavior-related cardiovascular risk factors have been associated with poor adherence to healthful dietary pattern. A recent analysis of the PREDIMED trial cohort at baseline, showed that little education, a larger waist-to-height ratio, diabetes, low physical activity, single, divorced or separated social status, and current smoking were associated with lower adherence to a MED diet [145]. Actually adherence could be one of the main barriers to the beneficial effect of the dietary approaches in CVD. A recent study by Wong and coll. showed that counseling towards choosing a DASH dietary pattern was not sufficient to obtain significant blood pressure reductions in a Chinese cohort of mild hypertensive patients, thus underscoring the importance of finding an efficient delivery model to get the expected benefits from an intervention [146]. In addition to the adherence issue, it is worth considering that potential benefits of some food categories may be outweighed by emerging drawbacks, as in the case of oily fish consumption and the risk associated with its content in toxic

lipophilic organic contaminants (e.g., organochlorins) and heavy metals (e.g., mercury) due to ocean pollution [147].

Awareness of the reasons of poor efficacy is mandatory for planning successful population interventions aimed at reducing the burden of CVD by targeting the main modifiable risk factor [3]. Potential interventions should not concentrate on single risk factors, but embrace a comprehensive approach and broad recommendations, to maximize results in large populations, as many studies and trials point out [148,149]. Knowledge of effective dietary patterns and recommendations should not be considered a finishing point: finding correct strategies to effectively deliver evidence-based dietary recommendations to the population should be considered as important as dietary knowledge. Integration of dietary approaches with effective health policies will provide a low-cost support to high-cost drugs and device strategies in helping to improve cardiovascular outcomes worldwide.

6. From Populations to Individuals: Towards a Tailored Diet Approach

Clinical trials and prospective studies of dietary patterns in CVD showed the effects of different diets on heterogeneous populations. Subsequently, subgroup analyses demonstrated that the effects of a dietary pattern vary among different subgroups of people according to sex, age, race, and other individual factors. Moreover, the main individual pathology, the presence of comorbidities and of different risk factors contributes to the different individual responses to a diet regimen. Beside these determinants of the response to different nutrient combinations there is another one, which is receiving growing attention: the individual genetic profile.

Nutrigenetics, an emerging branch among nutritional sciences, analyzes the interaction of diet with common gene variants of candidate genes that determine different responses to dietary interventions [150]. Significantly, several genes have been identified whose variants, when combined with various dietary inputs, determine different susceptibility to various conditions like dyslipidemias and atherogenesis [151], activation of inflammatory pathways [152], or diabetes and metabolic disease [153].

Recently, data from large feeding trials like the DASH and PREDIMED trials were analyzed and compared with known genes affecting CVD, to clarify whether there are genetic determinants of the efficacy of these dietary patterns, and eventually to determine if there are genetic responders and non-responders to these diets. In 2013, Corella and colleagues published the results of an analysis of Transcription factor 7-like 2 (TCF7L2) polymorphisms among the participants of the PREDIMED trial [154]. The product of TCF7L2 is a high-mobility box-containing transcription factor that plays a role in activating multiple genes, and the rs7903146C polymorphism (more than the rs7903146T) is one of the most influencing genetic variants for type 2 diabetes risk [155]. Notably, when adherence to the MED diet was low, TT homozygotes had higher fasting glucose concentrations (132.3 $\pm$ 3.5 mg/dL) than CC and CT individuals (127.3 $\pm$ 3.2 mg/dL, $p = 0.001$), but when adherence was high, this increase was not observed ($p = 0.605$). This modulation was also observed for total cholesterol, LDL-C, and TG (p interaction < 0.05 for all). Moreover, compared with CC, TT subjects had a higher stroke incidence in the control group (adjusted HR 2.91, 95% CI 1.36–6.19, $p = 0.006$), and dietary intervention with MED diet reduced stroke incidence in TT individuals (adjusted HR 0.96, 95% CI 0.49–1.87, $p = 0.892$) [154]. This provided evidence that a dietary pattern, the MED diet, can overrule the metabolic and cardiovascular genetic risk associated with individuals carrying particular genetic polymorphisms. Later, Ortega-Azorìn and colleagues showed that individual genetic profile could also act synergistically with a dietary pattern in determining beneficial effects. In a sample from the PREDIMED trial they observed that a variant (rs3812316) in the MLXIPL gene encoding the carbohydrate response element binding protein and associated with lower serum TG, had cumulative beneficial effects when combined with high adherence to the MED diet [156]. Additionally, a dietary pattern was shown to influence the development of CVD also by inducing changes in the individual transcriptomic response of genes involved in cardiovascular risk. In a small subset of subjects from the PREDIMED trial, Castañer and colleagues performed an analysis of multiple genes' expression

profile and observed that MED diet either supplemented with olive oil or mixed nuts induced a variation in the transcriptomic response modulating 12 of the 18 signaling pathways analyzed [157]. The influenced pathways were involved in cardiac hypertrophy, renin-angiotensin-aldosterone system (RAAS), nitric oxide signaling, atherogenesis, and cardiac β-adrenergic signaling. After adjustment, 9 pathways resulted modulated by one or both variants of the MED diet and none of the pathways remained modulated by the low-fat control diet.

For these reasons, knowledge of the individual genetic risk may be useful to target appropriate specific dietary interventions to override genetic risk or to favorably change the individual gene expression profile. Data supporting this novel aspect of dietary interventions comes also from the DASH trial. Recently, Chen and colleagues demonstrated that the DASH diet was associated with an increase in plasma renin activity (PRA) among subjects enrolled in the DASH trial [158]. This acts as a counter regulatory mechanism that blunts the BP lowering effect of this dietary pattern. The role of beta-2 adrenergic receptors (β2-AR) mediated vasodilation in response to adrenergic agonists and renin secretion in the juxtaglomerular cells has been extensively studied in past years [159,160]. Recently, Sun and colleagues analyzed in the DASH study population the G46A (Gly16Arg) variant of β2-AR, which is associated with impaired agonist mediated receptor downregulation and desensitization, low PRA, and salt-sensitive hypertension. Homozygosis for the A allele was associated with greater SBP reduction when combined with high adherence to the DASH diet whereas GG homozygotes showed no significant SBP change due to an increased PRA and aldosterone concentrations [161]. This study provides further evidence of a possible future application of patient genotyping to tailor dietary interventions to fit individual needs.

To summarize, a one-size-fits-all nutritional intervention may be a limited approach in patients with CVD. Indeed dietary requirements differ not only between primary and secondary prevention of CAD or HF, but also between different individuals. In the future, a personalized and tailored intervention may reach greater benefits, taking into account also individual genome, beside social geographical and cultural factors, the presence of CV risk factors, comorbidities and special needs related to the specific CV disorder (Figure 1).

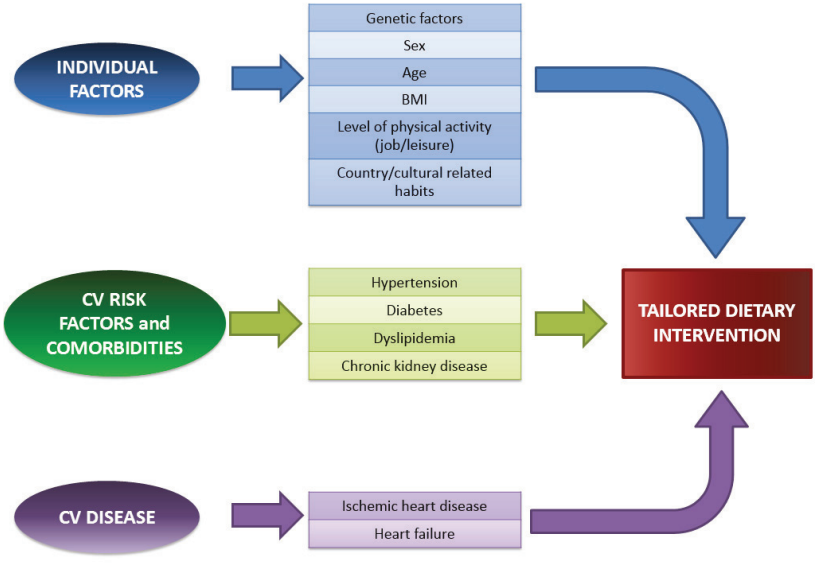

Figure 1. Factors to consider for tailoring dietary interventions in patients with CVD. BMI, Body Mass Index; CV(D), Cardiovascular (Disease).

Further investigations and well-designed clinical trials are warranted in this field in order to ascertain the clinical efficacy, the impact on outcomes and the cost-effectiveness of this kind of interventions, that may broaden the fan of available tools for the clinician, so that is possible to better reach different at-risk populations.

7. Conclusions

A dietary approach to nutritional interventions in CVD had proved to be an effective strategy resulting in strong and tangible results. The aforementioned studies indicate that synergistic effects of food combined into a dietary pattern provide the maximum benefit obtainable from nutrition. In the next years, further randomized trials may increase our knowledge on the effects of different food combinations on hard cardiovascular outcomes. This will certainly help Cardiologists and General Physicians in prescribing a "tailored" dietary pattern to each patient, thus providing a complimentary therapy acting together with drugs and devices to improve health and survival.

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Abbreviations

The following abbreviations have been used in the text:

AARP	American Association of Retired Persons
ACC	American College of Cardiology
AER	Albumin Excretion Rate
AHA	American Heart Association
β2-AR	Beta 2 Adrenergic Receptor
BMI	Body Mass Index
BP	Blood Pressure
CAD	Coronary Artery Disease
CARDIA	Coronary Artery Risk Development in Young Adults
CI	Confidence Interval
COR	Class Of Recommendation
CV	Cardiovascular
CVD	Cardiovascular Disease
DALY	Disability-Adjusted Life Year
DASH	Dietary Approach to Stop Hypertension
DBP	Diastolic Blood Pressure
EPIC	European Prospective Investigation into Cancer and nutrition
ESC	European Society of Cardiology
FFQ	Food Frequency Questionnaire
GLYNDIET	Glycemic Index of the Diet study
HALE	Healthy Ageing: a Longitudinal study in Europe
HDL (-C)	High Density Lipoprotein (Cholesterol)
HF	Heart Failure
HFpEF	Heart Failure with preserved Ejection Fraction
HFrEF	Heart Failure with reduced Ejection Fraction
HR	Hazard Ratio
LDL (-C)	Low Density Lipoprotein (Cholesterol)
LOE	Level of Evidence
LVEF	Left Ventricular Ejection Fraction
MED	Mediterranean

MESA	Multi-Ethnic Study of Atherosclerosis
MI	Myocardial Infarction
MLXIPL	Max-Like protein X Interacting Protein-Like
ND	Nordic Diet
NIH	National Institutes of Health
NT-proBNP	N-terminal fragment of the pro-peptide for Brain Natriuretic Peptide
NYHA	New York Heart Association
OMNI-Heart	Optimal Macro-Nutrient Intake Heart trial
PRA	Plasma Renin Activity
PREDIMED	PREvención con DIetaMEDiterránea
PUFA	Polyunsaturated Fatty Acids
QoL	Quality of Life
RAAS	Renin-Angiotensin-Aldosterone System
RR	Relative Risk
SBP	Systolic Blood Pressure
SCD	Sudden Cardiac Death
TCF7L2	Transcription Factor 7-Like 2
TG	Triglycerides
USDA	United States Department of Agriculture

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Internal Fat and Cardiometabolic Risk Factors Following a Meal-Replacement Regimen *vs.* Comprehensive Lifestyle Changes in Obese Subjects

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Abstract: The aim of the present study was to investigate the effect of a meal-replacement regimen *vs.* comprehensive lifestyle changes in overweight or obese subjects on intra-abdominal fat stores (Magnetic Resonance Imaging (MRI) measurements) and cardiometabolic risk factors. Forty-two obese men (n = 18) and women (n = 24) (age 49 $\pm$ 8 years; weight 96.3 $\pm$ 12.1 kg; BMI 32.7 $\pm$ 2.3 kg/m^2) were selected for this randomized parallel-group design investigation. Subjects in the lifestyle group (LS-G; n = 22) received dietary counselling sessions and instructions how to increase physical activity. In the meal replacement group (MR-G; n = 20) meals were replaced by a low-calorie drink high in soy protein. After six months, subjects in the LS-G lost 8.88 $\pm$ 6.24 kg and subjects in the MR-G lost 7.1 $\pm$ 2.33 kg; $p < 0.01$ for changes within groups; no significant differences were found between the groups. Lean body mass remained constant in both intervention groups. MRI analyses showed that internal fat was significantly reduced in both groups to a comparable amount; the higher fat loss in the LS-G in the abdominal area was due to a higher reduction in subcutaneous fat. Both interventions significantly reduced components of the cardiometabolic risk profile and leptin levels. The decrease in the adipokines fetuin A and resistin was more pronounced in the MR-G. In conclusion, both interventions significantly reduced body weight, total fat mass and internal abdominal fat while preserving lean body mass. The reduction in the adipokines fetuin A and resistin was more pronounced in the meal replacement group suggesting an additional effect of soy protein components.

Keywords: meal replacement; visceral fat; metabolic syndrome; lifestyle intervention

1. Introduction

Hypercaloric diets and sedentary behaviour are cornerstones in the development of obesity, the metabolic syndrome and type 2 diabetes mellitus. Hence, comprehensive lifestyle changes improve both, weight loss and metabolic risk factors.

Several lines of evidence suggest that one of the most important goals for lifestyle interventions is the reduction in fat mass and in particular the reduction in intra-abdominal/internal fat. It has been shown that cardiometabolic risk factors increase as a function of visceral fat accumulation [1]. A higher amount of visceral fat—relative to subcutaneous fat—is related to extra-adipocyte fat storage, reduced insulin sensitivity and increased pro-inflammatory adipokine concentrations. Therefore,

the appropriate measurement of the amounts of visceral fat and the ratio of visceral/subcutaneous fat is very important for the evaluation of the pathophysiological impact of total fat stores. Several measures of body composition routinely used in the clinic (e.g., waist circumference, waist/hip ratio or bioimpedance) have failed to predict visceral fat mass accurately [1].

Lifestyle interventions are effective in significantly reducing the amount of visceral fat. However, there is still an ongoing discussion which kind of lifestyle alteration is associated with the highest decrease in visceral fat and thus with the greatest benefit with respect to cardiometabolic risk factors [2–4].

The rational background for the present investigation was that although MR improve body weight and cardiometabolic risk factors [3,5,6], data on the respective effects on different fat stores is not available. MR regimens are criticized as associated with a loss in muscle mass favouring weight regain and that very low calorie diets predominantly reduce visceral fat in the early phase of weight loss interventions [4]. Moreover, little is known if the effects of a comprehensive lifestyle intervention (LSI) including aerobic exercise, psychological coaching and dietary intervention are superior to a MR regimen, particularly with respect to the influence on visceral fat [2].

2. Methods

In the present study, the effects of a six months meal replacement regimen (MR) on body composition and in particular on the amount of visceral and subcutaneous fat were investigated. In addition, body weight, metabolic risk factors and several adipokine concentrations were determined after six weeks and six months in order to document both short and longer term metabolic effects of the intervention. The results were compared to the respective effects of a comprehensive lifestyle intervention (LSI).

2.1. Subjects

Forty-two obese men (n = 18) and women (n = 24) (age 49 $\pm$ 8 years; weight 96.3 $\pm$ 12.1 kg; BMI 32.7 $\pm$ 2.3 kg/m^2) were recruited from the outpatient database of the University Hospital for this randomized parallel-group design investigation. The participants should be between the age of 18 and 65 years ($27 \leqslant$ BMI $\leqslant 40$) and capable to carry out a six month lifestyle intervention including physical exercise training.

Subjects with type 2 diabetes mellitus, clinically significant illnesses or patients who took anti-diabetic or lipid-lowering drugs were excluded. All subjects completed a comprehensive medical examination and routine blood tests. Written informed consent was provided by all subjects, and the study protocol was approved by the Ethical Committee of the University of Freiburg. Subjects were randomized into two equal groups as described previously using a random list [7]. The data presented here represent the pooled results of two studies that were performed identically in a comparable population group. The randomization process was done for each study separately and the pooling took place after completion of each study.

2.2. Intervention Program

The intervention in the lifestyle group (LS-G) consisted of 10 weekly teaching sessions related to nutrition, physical exercise and motivation. All sessions were held by certified experts in their respective fields. In addition, subjects received a hand-out with dietary advice and recommendations for lifestyle changes that were in accordance with the "German Society of Nutrition" and the "German Society of Sports Medicine and Prevention" [8]. The prescribed diet was a moderate-fat, nutrient-balanced weight reduction diet consisting of 1200 to 1500 kcal per day for women and 1500 to 1800 kcal per day for men, with approximately 50–55 percent of the calories coming from carbohydrates, preferably with a low GI, 25–30 percent from fat, and 15–20 percent from protein. Dietary behaviour was checked using two 24-h dietary recalls that were used for dietary compliance and that were individually discussed at the nutritional teaching sessions.

The subjects in the LS-G were instructed to increase physical activity according to the guidelines of the "German Society of Sports Medicine". They performed three physical activity sessions per week with an intensity ranging between 55% to 75% of VO_2max. In the first six weeks, physical activity was performed as a group session supervised by a physical education teacher two times/week; thereafter, the group sessions took place once a week. Participants were instructed to perform the other physical activity sessions independently.

The subjects assigned to the meal replacement group (MR-G) were instructed to replace two daily meals with a commercially available soy-yoghurt-honey preparation (Almased®) for the first six weeks. During the following 20 weeks, only one daily meal was replaced by the preparation. The dietary intake of fat during this second phase was not to exceed 60 g per day. The first six-week diet contained about 1000 kcal per day for women and 1200 kcal for men, and then, in the following 20 weeks, aimed at a maximum of 1500 kcal for women and 1700 kcal for men.

The data collected at enrolment and after six, and 26 weeks were body weight, waist and abdominal circumference, self-reported medical history, blood pressure, glucose, serum lipids and plasma levels of several adipokines. Leptin, resistin and fetuin A were measured by commercially available ELISA kits (DSL Deutschland GmbH, Sinsheim, Germany). All other laboratory analyses were done in the central laboratory of the University hospital using clinical routine methods. Waist circumference was taken with a non-distensible tape measure according to published guidelines [9]. At baseline and after 26 weeks, body composition analyses using the technique of air displacement plethysmography were performed (Bod Pod®, [10]).

2.3. MRI Measurements

MRI (Magnetic Resonance Imaging) measurements of total abdominal fat and the amount of subcutaneous and internal fat were determined at baseline and after 26 weeks. MRI measurements were performed on a 1.5 T short bore, whole-body MRI system with an inner diameter of 70 cm (Magnetom Espree, Siemens Healthcare, Erlangen, Germany). Data were acquired using a gradient echo based Dixon sequence as described previously [11]. The region of interest was covered by a multi-array spine coil in combination with two multi-array body coils.

Fat/Water MRI data were analysed after a two-point-Dixon fat water image reconstruction using an active contour snake segmentation to separate subcutaneous and internal adipose tissue, including visceral adipose tissue, muscular fat and bone marrow [11]. The classification follows the one used by Shen [12] and was done for the abdominal region ranging from the highest cranial extension of the liver (exhaled position) to the first cranial slice displaying the femoral heads.

2.4. Statistics

Normality of all variables was tested before statistical analyses using the Kolmogorov-Smirnov test procedure. Testing for changes between the examinations within the intervention groups was performed by applying the paired two-sample *T*-test. Multiple testing was considered using the Holm-Bonferroni method. Testing for changes between groups following the intervention (LS-G *vs.* MR-G) was done by using two-way repeated-measures analysis of variance (ANOVA) for continuous variables. The factors were treatment group (LS-G *vs.* MR-G) and time (levels were pre and post intervention (6 and 26 weeks).

All P values were two-sided and a *P* value of 0.05 or less was considered to indicate statistical significance. Analysis was conducted with the use of SPSS software (version 20.0.1, IBM, Armonk, NY, USA).

3. Results

As stated before, the data represent the pooled results of two studies that were performed in an absolute identical manner. In total, from the outpatient database of the University Hospital, 117 potentially eligible subjects were contacted and asked concerning exclusion criteria. Seventy-five

subjects were invited for screening and 50 subjects were eligible and randomized into the LS-G or MR-G. Forty-two patients completed the study and attended at least 75% of the meetings/training sessions. Eight participants dropped out: One subject changed the residence, five had claustrophobia, one subject was dissatisfied with the randomization outcome and one did not show-up again for follow-up for unknown reason.

For the final analysis, 22 subjects in the lifestyle group (LS-G) and 20 subjects in the meal replacement (MR-G) group were included.

3.1. Anthropometric Parameters

The changes in weight and BMI are shown in Table 1. After six weeks, the decrease in weight and BMI was more distinct in the MR group whereas after six months, weight loss was more pronounced in the LS group. At the end of the study, subjects in the LS-G had lost 8.88 $\pm$ 6.24 kg and subjects in the MR-G had lost 7.1 $\pm$ 2.33 kg. The changes within each group were highly significant ($p < 0.01$) whereas no significant differences could be observed between the groups. Figure 1 shows the results from the Bod Pod analysis indicating that the higher weight loss in the LS-G was due to a higher decrease in fat mass. Lean body mass did not change in either of the intervention groups. Figure 2 shows the results of the MRI scans. In the abdominal region, subjects in the LS group lost more fat than the MR group (A); however, the differences between the intervention groups were not significant for all MRI measures. Subjects in the MR-G exhibited lower subcutaneous and higher internal abdominal fat in the abdominal region at baseline. The latter findings could be explained by a higher proportion of females in the LS-G compared to the MR-G. However, although the female subjects in this investigation exhibited a higher mean subcutaneous fat mass (11.4 kg) than the male participants (8.6 kg), there were no distinct gender-related changes in the course of the intervention that could explain the different outcome in the LS-G *vs.* MR-G. Both, female and male subjects lost 2.9 kg of total abdominal fat and the surplus amount of subcutaneous fat loss was only 300 g in the female participants. In the LS-G, the surplus in subcutaneous fat loss was 1.4 kg (B) while internal fat loss was almost identical (C).

Table 1. Weight and BMI during the course of the study. Mean $\pm$ standard deviation (SD). LS-G, lifestyle intervention group; MR-G, meal replacement group. §, $p < 0.05$ compared to baseline; ◊, $p < 0.01$ compared to baseline (paired *T*-Test). Differences between the intervention groups (ANOVA) were not significant at any point of the investigation.

		Baseline	6 Weeks	6 Months	Changes after 6 Weeks	Changes after 6 Months
		Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
Weight (kg)	**LS-G**	95.9 (12.01)	92.1 (11.9) §	87.0 (12.2) ◊	−3.77 (2.85)	−8.88 (6.24)
	MR-G	96.7 (12.6)	91.3 (12.8) ◊	89.7 (13.1) ◊	−5.42 (1.86)	−7.06 (2.33)
BMI (kg/m^2)	**LS-G**	32.5 (2.65)	31.2 (2.62) §	29.4 (2.46) ◊	−1.28 (0.94)	−3.06 (2.13)
	MR-G	32.9 (1.88)	31.0 (2.08) ◊	30.5 (2.43) ◊	−1.85 (0.59)	−2.41 (0.77)

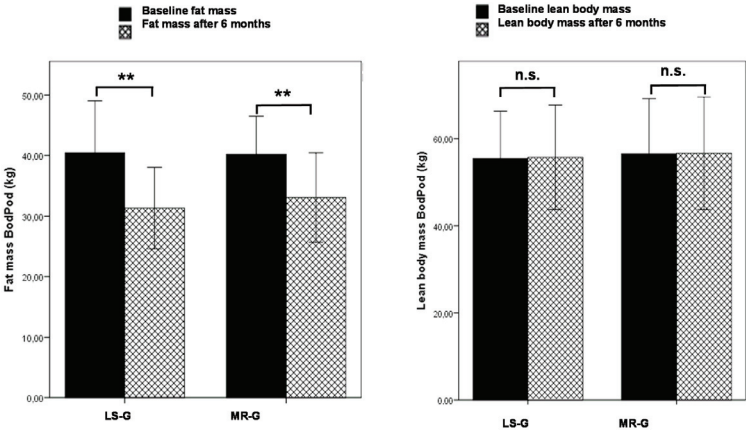

Figure 1. Changes in fat mass (PodBod) and lean body mass (PodBod) after six months by either lifestyle changes (LS-G) or a meal replacement regimen (MR-G); black bar, baseline values; grey hatched bars, post-interventional values after six months. (Error bars: ±1 standard deviation; *, $p < 0.05$; **, $p < 0.01$, n.s., not significant).

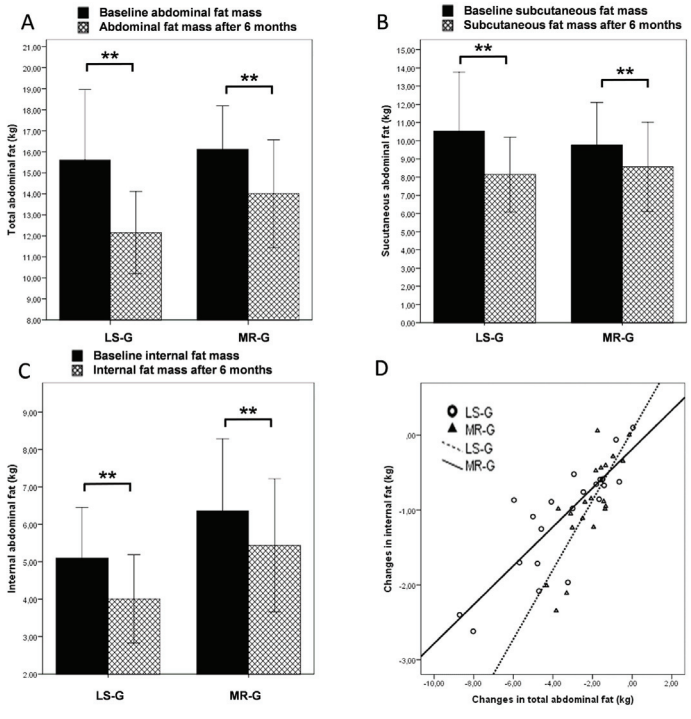

Figure 2. MRI findings: Changes in total abdominal fat mass (**A**); subcutaneous abdominal fat (**B**); internal abdominal fat (**C**) and the correlation between changes in total abdominal fat and internal abdominal fat in dependence of the intervention group (lifestyle changes LS-G or meal replacement regimen MR-G); black bars, baseline values; grey hatched bars, post-interventional values after six months. (Error bars: ±1 standard deviation; *, $p < 0.05$; **, $p < 0.01$).

Per kg fat mass lost during the intervention, participants in the MR-G lost more abdominal fat which is further illustrated by the scatterplot and the linear equation (D).

3.2. Cardiometabolic Risk Factors

Although there were no significant differences between the intervention groups at any time of the study, there were discrepant courses of parameters and differences in the significance levels within the two groups (Table 2). Total cholesterol decreased significantly after six weeks in both groups and rose again to near baseline levels after six months. Triglycerides levels were initially higher in the MR-G and decreased in both groups after six weeks; after six months triglycerides further decreased in the MR-G but rose again in the LS-G. After six month, triglycerides dropped by 51.4 $\pm$ 93.1 mg/dL in the MR-G and 19.4 $\pm$ 49.2 mg/dL in the LS-G. LDL-Cholesterol dropped significantly in both groups after six weeks with a more pronounced decline in the MR-G. After six months LDL-levels largely returned to baseline in both groups. Initial levels of glucose and HbA1c were higher in subjects in the LS-G. Glucose levels dropped in both groups but the decrease was only significant in the lifestyle group. HbA1c also dropped in both groups and after six months, the changes were significant in both groups. The changes were more distinct in the LS-G, however, comparable to glucose levels, initial HbA1c was also higher in subjects in the lifestyle group.

Table 2. Total cholesterol, triglycerides, LDL- and HDL-cholesterol, glucose and HbA1c levels during the course of the study. Mean $\pm$ standard deviation (SD). LS-G, lifestyle intervention group; MR-G, meal replacement group. §, $p < 0.05$ compared to baseline; ◊, $p < 0.01$ compared to baseline (paired *T*-Test). Differences between the intervention groups (ANOVA) were not significant at any point of the investigation.

		Baseline	6 Weeks	6 Months	Changes after 6 Weeks	Changes after 6 Months
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Cholesterol (mg/dL)	**LS-G**	225 (37.4)	202 (39.9) ◊	215 (33.4)	−23.7 (31.3)	−11.3 (30.1)
	MR-G	228 (38.5)	195 (31.4) ◊	223 (41.4)	−32.3 (24.3)	−4.71 (31.5)
Triglycerides (mg/dL)	**LS-G**	138 (61.4)	103 (37.4) ◊	118 (60.5)	−34.8 (51.1)	−19.4 (49.2)
	MR-G	172 (115)	125 (45.4) ◊	120 (41.9) ◊	−46.9 (88.2)	−51.4 (93.1)
LDL-Cholesterol (mg/dL)	**LS-G**	140.2 (40.4)	131 (34.6) §	139 (31.6)	−8.9 (35.3)	−0.51 (31.3)
	MR-G	147.2 (31.7)	127 (27.5) ◊	150 (36.1)	−20.3 (17.9)	3.27 (24.7)
HDL-Cholesterol (mg/dL)	**LS-G**	53.4 (14.7)	48.8 (8.99) ◊	54.6 (13.2)	−4.53 (10.9)	1.19 (9.17)
	MR-G	50.1 (9.42)	47.4 (7.87) §	52.3 (9.52) ◊	−2.65 (6.76)	2.25 (7.29)
Glucose (mg/dL)	**LS-G**	100.7 (25.1)	91.9 (14) §	92.2 (15.9) ◊	−8.70 (17.3)	−8.48 (15.1)
	MR-G	95.2 (19.9)	93.4 (11.9)	90.2 (10.6) #	−1.35 (10.7)	−4.55 (13.8)
HbA1c (%)	**LS-G**	5.64 (0.52)	5.51 (0.44) ◊	5.51 (0.38) ◊	−0.13 (0.23)	−0.13 (0.26)
	MR-G	5.58 (0.55)	5.52 (0.57)	5.50 (0.42) §	−0.06 (0.18)	−0.07 (0.34)

3.3. Adipokine Levels

None of the adipokines showed significant differences between the groups (Table 3). Plasma leptin levels decreased significantly and to a comparable degree in both groups. The reduction in resistin and fetuin A levels was more pronounced and only significant in the MR-G.

Table 3. Plasma levels of the adipokines Leptin, Resistin and Fetuin A during the course of the study. Mean ± standard deviation (SD). LS-G, lifestyle intervention group; MR-G, meal replacement group. §, $p < 0.05$ compared to baseline; ◊, $p < 0.01$ compared to baseline (paired *T*-Test).Differences between the intervention groups (ANOVA) were not significant at any point of the investigation.

		Baseline	6 Weeks	6 Months	Changes after 6 Weeks	Changes after 6 Months
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Leptin (µg(L)	**LS-G**	19.1 (11.9)	12.7 (8.39) ◊	9.85 (8.02) ◊	−7.91 (10.0)	−10.3 (11.3)
	MR-G	18.7 (19.3)	11.6 (10.9) ◊	9.36 (6.97) ◊	−5.70 (10.7)	−8.47 (13.2)
Resistin (µg/L)	**LS-G**	4.76 (1.68)	5.03 (1.58)	4.85 (1.88)	0.06 (0.78)	−0.13 (1.12)
	MR-G	5.53 (2.44)	4.67 (1.38) §	4.51 (1.35) ◊	−0.88 (1.64)	−0.88 (1.43)
Fetuin A (µg/mL)	**LS-G**	0.41 (0.1)	0.41 (0.1)	0.39 (0.09)	0.01 (0.08)	−0.01 (0.08)
	MR-G	0.38 (0.07)	0.36 (0.08) ◊	0.35 (0.08) §	−0.03 (0.06)	−0.03 (0.04)

4. Discussion

The main finding of the present study was that the meal replacement regimen decreased internal abdominal fat stores to the same degree as a comprehensive lifestyle intervention. In addition, total fat loss was not significantly different between the intervention groups, and both groups showed no decrease in fat free mass. The results regarding reductions in body weight and fat mass are in keeping with previous studies investigating the effect of meal replacements or comprehensive lifestyle interventions in overweight or obese subjects [13,14]. This investigation did not confirm the findings of a review by Chaston *et al.* that the preferential loss of internal fat by very low calorie diets disappears when the intervention period is longer than four weeks [4].

Nevertheless, albeit not significant, the lifestyle intervention further reduced total and abdominal fat stores. The results from the MRI-scans suggest that the additional fat loss in the lifestyle group could mainly be attributed to an increased fat loss from subcutaneous fat stores. This could not be explained by the higher proportion of women in the LS-G, since the gender-related differences following the intervention were small compared to the differences between the meal replacement and the lifestyle intervention. Although the evidence cannot be directly deducted from the design of the present investigation, it could be speculated that the greater weight loss is related to the additional training program in the lifestyle group. Previous studies have also shown that additional exercise did not further increase intra-abdominal fat loss when added to a hypocaloric diet [15,16].

However, although the individual amount and the intensity of physical exercise was supervised by the sports instructors, the exact duration and intensity of individual sports activities was not monitored.

Both interventions significantly reduced the metabolic risk profile. From a physician's perspective, the findings after six months had more relevance than the results from six weeks. Regarding changes with clinical significance, the reduction in triglycerides and increase in HDL-cholesterol was more pronounced in the meal replacement group whereas glucose and HbA1c-levels were lower in the lifestyle group. The latter finding could be due to higher baseline levels in the LS-G. Previous investigations have shown that meal replacement regimens induce rapid improvements in metabolic risk factors in subjects with the metabolic syndrome [17,18]. In the present investigation, the metabolic risk profile was relatively low, therefore, the improvements were also relatively small. However, the changes in both groups demonstrated that improvements could be accomplished and that the magnitude of beneficial effects increased as a measure of baseline levels.

Adipokine levels showed no significant differences between the groups during the course of the intervention. These adipokines were selected because they reflect the body's fat stores (leptin) and it has been speculated that they play an important role in the initiation and propagation of the

pro-inflammatory state associated with extra-adipocyte fat storage, insulin resistance, internal fat accumulation and atherosclerosis.

Leptin levels were comparably reduced in both groups whereas the reduction in resistin and fetuin A were more pronounced in the subjects in the MR-G. Although the levels of these adipokines were positively correlated with total fat mass and cardiometabolic risk factors (data not shown), the decrease was not directly correlated with a reduction in internal abdominal fat mass. It could be speculated that the higher reduction in fetuin A and resistin could be explained by the specific effects of soy protein. Some, albeit not all investigations have suggested a positive influence of soy protein, and in particular soy isoflavones, on proinflammatory adipokines, insulin resistance and body weight [19–21]. However, it has to be acknowledged that the design of the present investigation was not appropriate to establish a cause-effect relationship.

5. Conclusions

In conclusion, results from the present study have shown that both, lifestyle intervention by increased physical activity/hypocaloric low fat diet and meal replacement using a soy protein formula significantly decreased body weight, total fat mass and internal abdominal fat to a comparable amount while completely preserving lean body mass. Both interventions were associated with an improvement in metabolic risk factors although the effect of the intervention seemed to be dependent on pre-interventional levels. Although the effects in both groups were comparable with respect to internal abdominal fat mass, the reduction in adipokine levels was more pronounced in the meal replacement group suggesting an additional effect of soy protein related compounds e.g., genistein or isoflavones. This, however, needs to be further examined in forthcoming studies. It is important to emphasize that the long-term process of atherosclerosis cannot be influenced by short-term dietary modification. All interventions in obese subjects, particularly in patients with metabolic risk factors and increased atherosclerotic burden, should aim at inducing long-term modifications and not transient alterations in both, weight management and atherosclerotic risk factors.

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Author Contributions: Daniel Koenig was the principal investigator of the study. Peter Deibert, Martin Buechert, Denise Zdzieblik and Aloys Berg were involved in the design and execution of the study and performed the statistical analysis. All authors read and approved the final manuscript.

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Review

Dietary Capsaicin Protects Cardiometabolic Organs from Dysfunction

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Abstract: Chili peppers have a long history of use for flavoring, coloring, and preserving food, as well as for medical purposes. The increased use of chili peppers in food is very popular worldwide. Capsaicin is the major pungent bioactivator in chili peppers. The beneficial effects of capsaicin on cardiovascular function and metabolic regulation have been validated in experimental and population studies. The receptor for capsaicin is called the transient receptor potential vanilloid subtype 1 (TRPV1). TRPV1 is ubiquitously distributed in the brain, sensory nerves, dorsal root ganglia, bladder, gut, and blood vessels. Activation of TRPV1 leads to increased intracellular calcium signaling and, subsequently, various physiological effects. TRPV1 is well known for its prominent roles in inflammation, oxidation stress, and pain sensation. Recently, TRPV1 was found to play critical roles in cardiovascular function and metabolic homeostasis. Experimental studies demonstrated that activation of TRPV1 by capsaicin could ameliorate obesity, diabetes, and hypertension. Additionally, TRPV1 activation preserved the function of cardiometabolic organs. Furthermore, population studies also confirmed the beneficial effects of capsaicin on human health. The habitual consumption of spicy foods was inversely associated with both total and certain causes of specific mortality after adjustment for other known or potential risk factors. The enjoyment of spicy flavors in food was associated with a lower prevalence of obesity, type 2 diabetes, and cardiovascular diseases. These results suggest that capsaicin and TRPV1 may be potential targets for the management of cardiometabolic vascular diseases and their related target organs dysfunction.

Keywords: chili pepper; capsaicin; TRPV1; metabolic syndrome; obesity; hypertension; diabetes

1. Introduction

A lot of protective natural compounds had been found for their neuroprotective properties in preventing diseases and inflammation [1–4]. Chili peppers have become a vital part of culinary cultures worldwide and have a long history of use for flavoring, coloring, and preserving food, as well as for medical purposes. Although some people are intolerant to pungency because of the sensation of heat and pain in the oral cavity, as well as varying degrees of gastrointestinal side effects, there remain many loyal consumers of this original South American plant. The increased use of chili peppers in food is a major trend around the world [5]. Capsaicin, the pungent ingredient in chili peppers, is an indispensable condiment, and it has shifted from an industrialized purified product to a daily nutrient. The beneficial effects of capsaicin have been validated in experimental and population studies.

The receptor for capsaicin is called the transient receptor potential vanilloid subtype 1 (TRPV1). TRPV1 belongs to the transient receptor potential (TRP) family, which is a heterogeneous group of non-selective cation channels. Based on their structural homology, mammalian TRP channels can be divided into six subfamilies, including the TRP canonical (TRPC; TRPC1–7), TRP vanilloid (TRPV; TRPV1–6), TRP melastatin (TRPM; TRPM1–8), TRP mucolipin (TRPML; TRPML1–3), TRP

ankyrin (TRPA; TRPA1), and TRP polycystin (TRPP; TRPP2, TRPP3, TRPP5) subfamilies [6]. It is well documented that TRP channels are involved in visual, auditory, taste, and pain signal transduction pathways. Emerging evidence indicates that TRP channels also participate in the regulation of cell survival and growth, mineral absorption, body fluid balance, gut motility, and cardiovascular function [7]. TRPV1 is a highly investigated TRPV subfamily member. In addition to its classical role in the nervous system, TRPV1 plays important roles in the maintenance of physiological homeostasis. Capsaicin is passively absorbed with greater than 80% efficiency in the stomach and upper portion of the small intestine and is transported by albumin in the blood [8]; therefore, it may extensively activate local TRPV1 channels in different organs or tissues to initiate a series of physiological effects.

2. Physiological Function of TRPV1

TRPV1 is widely expressed in the brain, sensory nerves, dorsal root ganglia, bladder, gut, and blood vessels [9,10]. TRPV1 is a ligand-gated non-selective cation channel, which is activated by multiple stimuli, including heat (>43 °C), voltage, low pH (<5.9), endogenous lipid molecules, and exogenous agonists, such as capsaicin [11] (Figure 1). Activation of TRPV1 leads to increased intracellular calcium levels and various physiological effects [12].

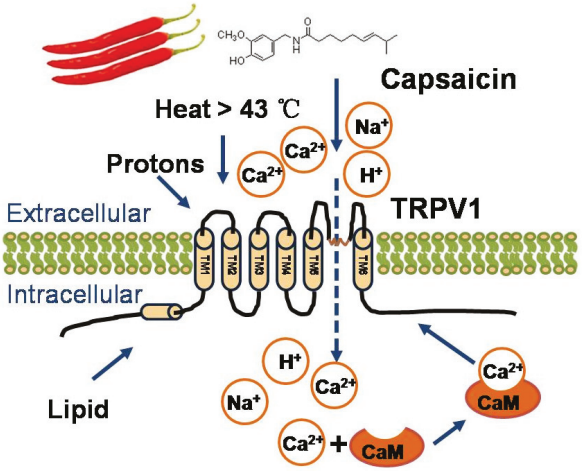

Figure 1. Structural and physiological function of TRPV1. TRPV1 is composed of six transmembrane domains. It has a short, pore-forming hydrophobic stretch between the fifth and sixth transmembrane domains. TRPV1 is activated by noxious heat (>43 °C), acid (pH < 5.9), voltage, and various lipids. Additionally, capsaicin activates TRPV1 and triggers cation influx and various subsequent physiological processes.

TRPV1 has prominent roles in inflammation, oxidative stress, and pain sensation [13]. Recently, emerging evidence suggests that TRPV1 also plays a critical role in the regulation of cardiovascular function and metabolic homeostasis. Activation of TPRV1 by its specific agonist capsaicin promotes endothelium-dependent vasodilation and subsequently contributes to lower blood pressure [14]. TRPV1 activation *in vivo* or in isolated perfused kidneys may increase the glomerular filtration rate and enhance renal sodium and water excretion [15,16]. TRPV1 was shown to be a potential target for the prevention of obesity because of its effect on energy balance [17,18]. Several studies found that activation of TRPV1 by capsaicin attenuated abnormal glucose homeostasis by increasing insulin secretion, insulin responses, and glucagon-like peptide 1 levels [19–21]. Furthermore, TRPV1 was shown as a regulator of growth factor signaling in the suppression of tumorigenesis [22], and its anti-cancer effect was also confirmed [23–25]. Furthermore, the TRPV1 receptor can be desensitized with high administration of

capsaicin in nervous tissue [26], but whether this effect exists in cardiometabolic tissues was never tested. Furthermore, capsaicin also plays its effects in a receptor-independent manner. It reported that capsaicin could inhibit NF-kappa B and modulate adipocyte function in obese-mouse adipose tissues and isolated adipocytes which is independent on TRPV1 [27].

3. Roles of TRPV1 in Cardiometabolic Diseases

Mounting evidence indicates that TRPV1 activation by capsaicin is beneficial for the management of obesity, diabetes mellitus, cardiovascular diseases, various cancers, dermatological conditions, and neurogenic bladder [14,19,22,28,29].

3.1. Activation of TRPV1 by Capsaicin Prevents Obesity

Obesity is involved in the development of obesity-related disorders, such as diabetes, hyperlipidemia, fatty liver, and cardiovascular diseases. The results from both human and animal studies indicate that TRPV1 and its agonist capsaicin are involved in energy expenditure and may represent a potential strategy to treat obesity. Capsaicin inhibits obesity by regulating energy metabolism, reducing adipose tissue weight, and increasing lipid oxidation [17]. However, the underlying mechanisms are not fully understood.

Brown adipose tissue (BAT) is prominent in the regulation of energy expenditure and body fat [30,31]. TRPV1 activation by capsaicin can activate sympathetically-mediated BAT thermogenesis and reduces body fat [31]. Intragastric administration of capsiate, another TRPV1 agonist, also resulted in a time- and dose-dependent increase in integrated BAT sympathetic nerve activity and an increased the metabolic rate [32]. Capsinoids were found to increase the metabolic rate and enhance thermogenesis via gastrointestinal TRPV1 [33]. Endogenous TRPV1 ligands reduced food intake in wild-type mice but not in TRPV1-null mice [34]. The recruitment of catecholaminergic neurons by TRPV1 activation also contributed to the extra energy expenditure [35]. The results of a proteomic analysis revealed that approximately 23 protein spots, which are related to thermogenesis and lipid metabolism, were significantly altered in a capsaicin-fed rat liver. These proteins may represent potential targets of capsaicin to attenuate obesity [36]. Adipogenesis is the critical and original process of fatty adipose accumulation. Zhang *et al*. found that capsaicin treatment inhibited adipogenesis of 3T3-L1-preadipocytes *in vitro* and prevented high fat diet induced obesity [28]. Moreover, chronic activation of TRPV1 by dietary capsaicin reduced lipid deposition in the liver and alleviated abdominal obesity [37]. The mechanism of action was related to upregulated uncoupling protein 2 (UCP2) expression in hepatocytes [37]. Capsaicin treatment-enhanced lipolysis was associated with increased levels of hormone sensitive lipase, carnitine palmitoyl transferase-Iα and UCP2 [38]. Peroxisome proliferation activated receptor (PPAR) α, a key regulator of glucose and lipid metabolism, was also involved in the capsaicin treatment-induced decrease of levels of inflammatory cytokines and lipid droplet accumulation in the liver [39]. Dietary capsaicin supplementation in mice fed a high-fat diet confirmed that PPARγ expression in adipose tissue was decreased, whereas weight gain and visceral fat mass were blunted [12]. Similarly, Lee *et al*. showed that after topical application of capsaicin cream to the skin of mice fed a high-fat diet for 8 weeks, the mesenteric adipose tissue weighed less than that of the control obese mice [38]. The levels of plasma glucose, cholesterol, and triglycerides were also lower in the capsaicin-treated mice [38]. These beneficial effects of capsaicin treatment were associated with up-regulated adipokines, such as adiponectin and leptin [38].

The role of the TRPV1 channel deletion in obesity is controversial. Motter *et al*. [40] found that TRPV1-null mice exhibited a significantly greater thermogenic capacity. It suggested that inhibition of TRPV1-sensitive sensory seems to be protective. Lee *et al*. [41] demonstrated that TRPV1-null mice became more obese than wild-type mice while consuming a high-fat diet. The discrepancy between these findings could be associated with the different composition of high-fat diet (55% kcal% fat *vs*. 25.8% kcal% fat), feeding regimen, and the intervention time.

3.2. Activation of TRPV1 by Capsaicin Improves Glucose Homeostasis

Diabetes mellitus is one of the most important public health challenges. The prevention and treatment of diabetes mellitus, as well as a reduction in its microvascular and macrovascular complications, requires not only pharmacological approaches, but also a major integrated approach directed at societal and individual behavioral change. As a natural material and food ingredient, capsaicin has been extensively investigated because of its role in improving glucose homeostasis and alleviating diabetes.

The pathogenesis of type 2 diabetes is complex and involves oxidative stress, endoplasmic reticulum stress, and inflammation, facilitating insulin resistance and beta cell dysfunction. TRPV1 may promote insulin secretion via its calcium influx activity in β-cells; however, the mechanism by which TRPV1 affects insulin synthesis, degradation, and secretion is unknown [42]. Activation of TRPV1 alleviates insulin resistance and regulates glucose homeostasis by suppressing inflammation. Dietary capsaicin reduced obesity-induced insulin resistance and leptin resistance in mice [39,41]. This beneficial effect of capsaicin was due to the attenuation of inflammatory phenotypes and enhanced adiponectin expression in adipose tissue and liver, which are important peripheral tissues for insulin sensitivity [39]. Subsequently, dietary capsaicin significantly decreased fasting glucose/insulin and triglyceride levels, as well as the expression of inflammatory adipocytokine genes [43]. Moreover, insulin sensitivity during hyperglycemic states was enhanced in diabetic rats on a capsaicin diet [44]. Our previous studies showed that capsaicin supplementation ameliorates abnormal glucose homeostasis in diabetic mice via stimulating GLP-1 secretion [19]. Chronic capsaicin supplementation not only improved glucose tolerance and increased insulin levels but also lowered the daily blood profiles and increased plasma GLP-1 levels [19]. Both capsaicin and capsiate treatment reduced body weight gain, visceral fat accumulation, and serum leptin levels, and improved glucose tolerance without modulating energy intake in diabetic rats [44]. Both also protected β-cell mass by increasing proliferation and decreasing apoptosis [44]. As an exogenous agonist of TRPV1, capsaicin is a potential target for the management of type 2 diabetes.

3.3. Activation of TRPV1 by Capsaicin Alleviates Hypertension

As one of the leading risk factors for cardiovascular disease, the pathogenesis of hypertension refers to the imbalance between vasoconstriction and vasodilatation. Intracellular Ca^{2+} homeostasis is essential for vascular function and blood pressure regulation [45]. Disturbance of Ca^{2+} homeostasis contributes to vascular dysfunction and high blood pressure [46,47]. TRPV1 is involved in hypertension and its related target organ dysfunction.

Activation of TRPV1 by capsaicin exerts an anti-hypertension effect by promoting the release of calcitonin gene-related peptide (CGRP) from capsaicin-sensitive nerves and nitric oxide (NO) from endothelial cells. Acute administration of capsaicin induced a transient CGRP increase in the plasma and was accompanied by a decrease in blood pressure [48]. Yang *et al*. reported that activation of TRPV1 by dietary capsaicin up-regulated the phosphorylation of PKA and eNOS and, therefore, the bioavailability of NO in endothelial cells [14]. Long-term capsaicin treatment enhanced endothelium-dependent relaxation and lowered blood pressure in genetically hypertensive rats [14]. In addition, the release of CGRP contributed to the hypotensive effect of chronic capsaicin consumption, but to a lesser degree [14]. Recently, the inhibition of L-type Ca^{2+} channels in rat aortic smooth muscle cells was identified as the mechanism underlying capsaicin-induced relaxation [49]. TRPV1 activation prevented the salt-induced increase in blood pressure in Dahl salt-resistant rats [50]. However, the expression and function of TRPV1 were compromised in Dahl salt-sensitive rats, which rendered the Dahl salt-sensitive rats susceptible to salt load in terms of blood pressure regulation [50]. Chronic dietary capsaicin ameliorated excess salt consumption-induced vascular dysfunction and nocturnal hypertension by inhibiting vascular oxidative stress in a TRPV1-dependent manner [51]. Urinary sodium excretion was much higher in mice on a high salt diet plus capsaicin supplementation compared to mice fed only a high-salt diet [52]. This natriuretic effect of TRPV1 activation by

capsaicin contributed to the lower blood pressure in mice fed a high salt diet. Furthermore, the general aversive behavior to salt caused by TRPV1 contributed to a lower dose of salt intake [53]. Marshall *et al*. [54] showed that TRPV1 deletion could protect against obesity-induced hypertension. Our studies demonstrated that the activation of TRPV1 by dietary capsaicin can attenuate genetic and high-salt diet induced hypertension [14,52]. Thus, the differences in experimental design and interventions could be responsible for this discrepancy.

3.4. Activation of TRPV1 Antagonizes Dysfunction of Cardiometabolic Organs

Dietary capsaicin has favorable effects on obesity, diabetes, hypertension, and metabolic syndrome. Conceivably, activation of TRPV1 may alleviate cardiometabolic organs dysfunction, including ameliorating atherosclerosis, cardiac hypertrophy, non-alcoholic fatty liver, and stroke risk (Figure 2).

Capsaicin-rich diets have been found to improve lipid metabolism, and capsaicin supplementation reduced diet-induced hypertriglyceridemia in rodents [37,55]. Activation of TRPV1 by capsaicin-ameliorated non-alcoholic fatty liver disease in mouse models [37], and TRPV1-mediated induction of PPARδ and UCP2 likely played a role in this effect [37]. The lipoprotein lipase activity was higher in adipose tissues following capsaicin administration [56]. Dietary capsaicin also slows atherogenesis, an effect that may reflect a favorable impact of TRPV1 activation on foam cells. Ma *et al*. found that TRPV1 activation significantly inhibited foam cell formation by increasing ATP-binding cassette transporter A1 expression and reducing low-density lipoprotein-related protein 1 expression [57]. Chronic activation of TRPV1 by capsaicin supplementation reduced atherosclerotic lesions in the aorta from high-fat diet fed ApoE−/− mice but not from ApoE−/−TRPV1−/− mice [57]. The liver X receptor α also played a critical role in TRPV1-activation-conferred protection against oxLDL-induced lipid accumulation and TNF-α-induced inflammation in macrophages [58]. Recently, we showed that TRPV1 activation antagonized coronary lesions by alleviating endothelial mitochondrial dysfunction and enhancing the activity of the PKA/UCP2 pathway [59]. Ultimately, this beneficial effect of TRPV1 activation prolonged the mean survival time of atherosclerotic mice [59].

In the heart, TRPV1 activation blunted cardiac hypertrophy and fibrosis [60,61]. High-salt intake-induced cardiac hypertrophy and fibrosis were characterized by a significant enhancement of heart weight, decreased heart function, and increased collagen deposition [60]. These alterations were related to the downregulation of PPARδ and UCP2 expression, the upregulation of iNOS production, and increased oxidative/nitrotyrosine stress [60]. Oxidative phosphorylation and the enzyme activity of the mitochondrial complex I were impaired in TRPV1 knockout or high-salt diet fed mice [61]. Indeed, these adverse effects of long-term high-salt intake were blunted by chronic capsaicin supplementation in a TRPV1-dependent manner [60,61]. Capsaicin-rich diets also attenuated pressure overload- and angiotensin II-induced cardiac hypertrophy and fibrosis [62].

Dietary capsaicin was shown to significantly delay the onset of stroke and increase the survival time of spontaneously hypertensive stroke-prone rats [63]. This anti-stroke effect of capsaicin was related to the enhanced relaxation of cerebral arteries and reversed hypertrophy of cerebral arterioles [63]. The neuroprotective effects of endocannabinoids were partially mediated by TRPV1, which afforded protection to the blood-brain barrier during ischemic stroke [64].

Diabetic vascular complication is a major cause of death and disability. Diabetes mellitus induces vascular endothelial dysfunction via several mechanisms, including excessive ROS generation following mitochondrial disturbance and the impairment of eNOS activity, finally leading to oxidative stress and endothelium dysfunction. UCP2 is a physiological regulator of mitochondrial ROS generation and may contribute to the prevention of diabetes and its related complications. Sun *et al*. found that upregulation of UCP2 by capsaicin decreased ROS production and increased NO bioavailability [65]. Chronic dietary capsaicin suppressed vascular oxidative stress and improved endothelium-dependent relaxation in diabetic mice [65]. By contrast, the expression of TRPV1 was decreased in diabetic mesenteric arteries, which was associated with impaired capsaicin-induced vasodilatation [66].

The gastrointestinal tract releases various gut hormones, such as cholecystokinin, ghrelin, peptide YY, and GLP-1, which participate in the stimulation of gastrointestinal function, the maintenance of energy homeostasis and metabolism [67]. Recently, several studies reported that gut hormones are involved in the pathogenesis of diabetes, obesity and hypertension [68–70]. Dietary capsaicin stimulated the intestinal mucosal afferent nerves and increased intestinal blood flow, which may affect the physiological function of the gastrointestine [71]. GLP-1 plays a principal role in the regulation of glucose metabolism by modulating insulin secretion and activating the gut-brain-periphery axis [72]. We found that TRPV1 receptors are present in GLP-1-expressing intestinal cells and that activation of TRPV1 stimulated GLP-1 release via a Ca^{2+}-dependent mechanism [19]. Chronic dietary capsaicin lowered blood glucose levels and improved glucose homeostasis in db/db mice [19]. Human studies also demonstrated that a single meal with capsaicin increased plasma GLP-1 concentrations and tended to decrease the plasma ghrelin concentrations during the postprandial phase [73]. Ghrelin is a stimulator of food intake and a centrally-acting orexigenic hormone. Activation of the capsaicin-sensitive vago-vagal reflex pathway was involved in ghrelin stimulated gastric motility [74]. Nesfatin-1, a newly identified hormone, belongs to a family of anorexigenic peptides. Nesfatin-1-induced protection was attenuated by pretreatment with the TRPV1 receptor inhibitor capsazepine [75].

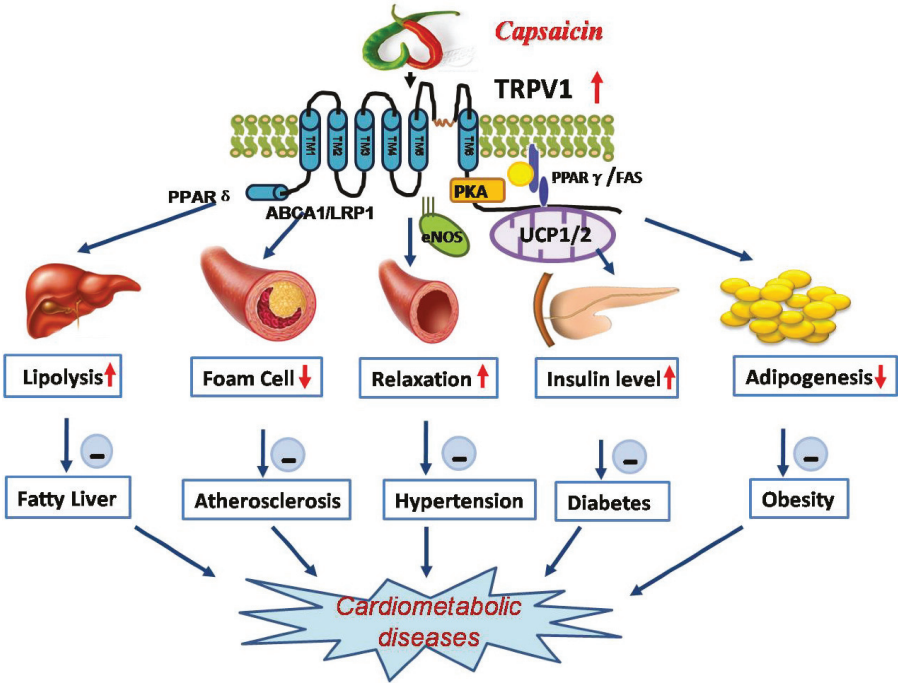

Figure 2. Favorable effects of capsaicin on cardiometabolic disease or related target organ damage. Activation of TRPV1 by dietary capsaicin plays a critical role in the regulation of lipid and glucose metabolism and vascular function, including the promotion of lipolysis by activating PPARδ, the improvement of vasodilation by increasing eNOS expression, the upregulation of insulin levels by activating PKA, the inhibition of foam cell formation by regulating ABCA1 and LRP1 levels, and the suppression of adipogenesis by activating PPARγ. Therefore, dietary capsaicin can alleviate fatty liver, atherosclerosis, hypertension, diabetes, and obesity. Dietary capsaicin has potential benefits for cardiometabolic diseases in the population.

4. Beneficial Effects of Dietary Capsaicin Consumption in Humans

The favorable effects of spices and their bioactive ingredients, such as capsaicin, have been documented in many experimental studies. Population studies also confirm the beneficial effects of capsaicin on human health (Table 1).

In a large prospective cohort study, the habitual consumption of spicy foods was inversely associated with both total and certain cause-specific mortality among both men and women after adjustment for other known or potential risk factors [76]. Inverse associations were also observed for deaths due to cancer, respiratory diseases, and ischemic heart diseases [76]. Compared with people who ate spicy foods less than once a week, the people who ate spicy foods almost every day had a 14% lower risk of death [76].

Epidemiologic data also showed that the consumption of foods containing capsaicin is associated with a lower prevalence of obesity, type 2 diabetes, and cardiovascular diseases [77,78]. Obesity is prominent risk factor for diabetes and cardiovascular diseases. Increased energy expenditure, enhanced lipid oxidation, and reduced appetite are potentially beneficial for weight management. Dietary red pepper was shown to suppress energy intake and modify macronutrient intake through its effects on appetite and energy expenditure. The ingestion of red pepper decreased appetite and subsequent protein and fat intake [79]. Red pepper also increased diet-induced thermogenesis and lipid oxidation [80]. The consumption of capsaicinoids increased energy expenditure by approximately 50 kcal/day [13], and this would produce clinically significant levels of weight loss [81]. However, there were inconsistent results for these outcomes. Some studies did not show any effect on substrate oxidation, energy expenditure or appetite following one meal or long-term administration in the form of capsules, juice, or supplements [82]. These differences may be a result of race, district, and dietary habits. Additionally, the range of dosage, method of administration and composition are widely varied. Therefore, a dietary capsaicin test is in urgent need of a unified standard.

The potential role of capsaicin in glucose homeostasis has been validated in animal experiments, although evidence for capsaicin regulating glucose metabolism in humans is relatively limited. In a large prospective study, the inverse association of daily spicy food consumption with death due to diabetes was observed in people who habitually ate fresh chili peppers [76]. In healthy human subjects, capsaicin treatment increased glucose absorption from the gastrointestinal tract and increased glucagon release during glucose loading tests [83]. The increased release of glucagon was proposed to be independent of insulin release after glucose loading. A low dose of capsaicin stimulated glucose absorption from the gastrointestinal tract in healthy human subjects and promoted the mobilization of glycogen via the stimulation of capsaicin-sensitive afferent nerves [83]. This result indicates that capsaicin-sensitive afferent nerves exert an important role in glucose utilization. Furthermore, topical capsaicin treatment significantly relieved pain at 12 weeks in patients with diabetic peripheral neuropathy [84].

Whether capsaicin is favorable for human cardiovascular health needs further study. The habitual consumption of spicy foods was inversely associated with deaths due to ischemic heart diseases [76]. Capsaicin may inhibit ADP-induced platelet aggregation [85,86]. The transdermal administration of capsaicin improved the ischemic threshold and exercise time in patients with stable coronary disease [87]. NO levels were also increased in the blood, and NO-mediated vasodilation may contribute to this clinical benefit. In combination with isoflavone, capsaicin significantly reduced both systolic and diastolic BP in hypertensive patients with alopecia [88]. This was likely associated with elevated serum levels of insulin-like growth factor-I, which has an antihypertensive effect [88].

Capsiate, a recently identified non-pungent capsaicin analog, presents a promising alternative for those who abstain from capsaicin-containing foods due to pungency. It provides a more acceptable compound for clinical trials that study the potential mechanism of dietary capsaicin on cardiometabolic organ protection in the population [89].

Table 1. The effects of capsaicin on cardiometabolic diseases in human studies.

Involved Diseases	References	Effect of Capsaicin	Underlying Mechanism
Obesity	[79–81]	+	increase energy expenditure, lipid oxidation and sympathetic nervous system activity; decrease appetite and subsequent protein and fat intake
	[82]	N	-
Type 2 diabetes	[83]	+	increase glucose absorption and glucagon release
Diabetic peripheral neuropathy	[84]	+	stimulation of capsaicin-sensitive afferent nerves
Cardiovascular diseases			
Coronary disease	[86,87]	+	inhibit ADP-induced platelet aggregation;
	[88]		increase NO levels in the blood and NO mediated vasodilation
Hypertension		+	elevate serum levels of insulin-like growth factor-I

Note: +, beneficial effect; N, no effect. [79–81,86,87]: randomized controlled trial, [82]: meta-analysis, [83]: comparative study, [84]: randomized, double-blind and parallel-group trial, [88]: case-control studies.

5. Conclusions

Capsaicin is not only a dietary nutrient but also a natural bioactive food ingredient. Capsaicin is involved in thermogenesis, lipid metabolism, the inflammatory response, and oxidative stress. These effects of capsaicin reduce adipogenesis, alleviate insulin resistance, ameliorate vascular dysfunction and regulate glucose homeostasis. These pathophysiologic processes are responsible for the pathogenesis of cardiometabolic diseases, such as obesity, hypertension, dyslipidemia, diabetes and atherosclerosis. Capsaicin plays a potential role in cardiometabolic protection through the activation of TRPV1 in different target organs or tissues, which suggests that TRPV1 may be a promising target for the management of cardiometabolic diseases. It is necessary to identify a more acceptable way to clarify the association between the dosage of dietary capsaicin and the effect on cardiometabolic protection to finally reach a consensus on the daily usage of capsaicin or its derivatives.

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Review

Caloric Restriction as a Strategy to Improve Vascular Dysfunction in Metabolic Disorders

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Abstract: Caloric restriction (CR) has proved to be the most effective and reproducible dietary intervention to increase healthy lifespan and aging. A reduction in cardiovascular disease (CVD) risk in obese subjects can be already achieved by a moderate and sustainable weight loss. Since pharmacological approaches for body weight reduction have, at present, a poor long-term efficacy, CR is of great interest in the prevention and/or reduction of CVD associated with obesity. Other dietary strategies changing specific macronutrients, such as altering carbohydrates, protein content or diet glycemic index have been also shown to decrease the progression of CVD in obese patients. In this review, we will focus on the positive effects and possible mechanisms of action of these strategies on vascular dysfunction.

Keywords: caloric restriction; dietary intervention; endothelial dysfunction; cardiovascular disease; obesity

1. Introduction

Obesity is a chronic disease due to an energetic imbalance in which caloric intake is higher than energetic expenditure. It is closely associated with insulin resistance and type 2 diabetes (T2D), leading to several manifestations of cardiovascular disease (CVD), such as hypertension, coronary artery disease, myocardial infarction, heart failure and stroke [1]. CVD is a major health problem worldwide accounting for 30% of all deaths and demands a global approach to prevention and early detection. In obese individuals, the earliest indication of vascular dysfunction preceding the development of prehypertension and hypertension is the impairment of endothelial function [2]. Therefore, endothelial function improvement should become a key approach to prevent and/or treat cardiovascular (CV) complications related to metabolic disorders.

The primary goal to reduce CVD risk in subjects who are overweight and obese is a moderate and sustainable weight loss. An improvement of multiple metabolic and hormonal factors implicated in the pathogenesis of CVD associated with metabolic disorders is already achieved by a weight loss of 5%–10% [3] that needs to be maintained over time. Since pharmacological approaches for body weight (BW) reduction have, at present, a poor long-term efficacy, other strategies such as caloric restriction (CR), exercise programs, or bariatric surgery are of great interest [3,4].

CR is defined as a state in which energy intake is reduced below usual *ad libitum* intake without malnutrition, independently of its duration. It is one of the most common and cost-effective interventions used to induce BW reduction and CV risk factor amelioration. Other dietary strategies changing specific macronutrients have also been shown to decrease progression of CVD. It is important to note that the induction of a negative energy balance is mandatory for achieving the metabolic benefits of weight loss since the sole reduction in fat mass alone by surgical procedures does not improve CV risk factors in obese patients [5,6]. Taking into consideration the complex and vast

literature regarding dietary strategies, in this review, we will focus on the positive effects on vascular dysfunction, CV risk factors and CVD exerted by CR and macronutrients intake modification.

2. CR Reduces CV Risk Factors

Benefits on CV risk factors by reducing the daily caloric intake have been widely described in overweight and obese patients [7–19]. CR induces reductions in BW, waist perimeter, total fat, serum triglycerides (TG), or low-density lipoprotein (LDL)-cholesterol concentrations [12,14,20]. It is also associated with a reduction of circulating insulin levels together with an increase in insulin sensitivity [12,14,18,20]. The decrease in adiposity leads to reductions in leptin [14,18], inflammatory cytokines, prostaglandins, and/or oxidative stress [9,10,14,18], as well as to an increase in the anti-inflammatory Il-10 [14] and adiponectin (Figure 1) (see Section 4.4). In obese patients with or without associated hypertension, weight loss enhances flow-mediated vasodilation (FMD, which determines endothelial function *in vivo*) [8,11], and induces a reduction in blood pressure (BP) [7,20].

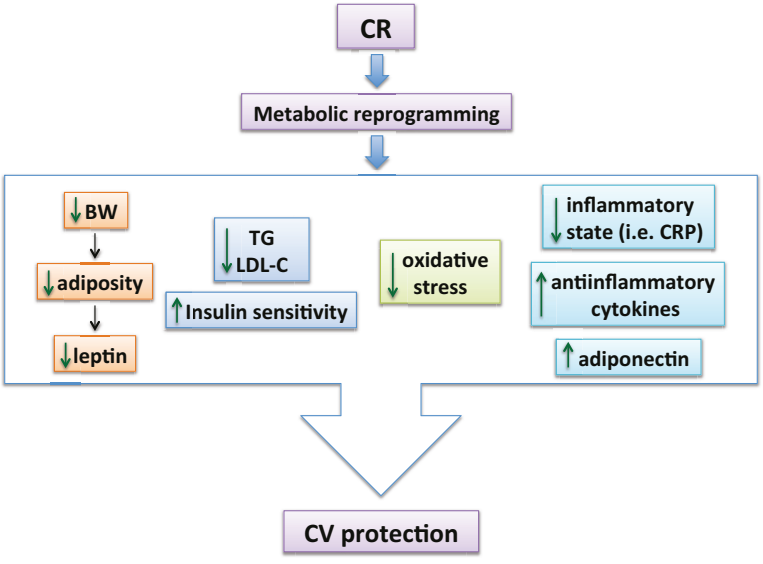

Figure 1. Main mechanisms by which CR exerts CV protection. Reducing the daily caloric intake induces a metabolic reprogramming in both healthy and obese individuals, subsequently leading to CV protection. This includes a reduction in BW and adiposity, thus lessening leptin levels. A decrease in TG and LDL-cholesterol levels together with less oxidative stress and inflammation and an increase in adiponectin levels are some of the main underlying mechanisms described (BW: body weight; CR: caloric restriction; CRP: high-sensitivity C-reactive protein; CV: cardiovascular; LDL-C: low-density lipoprotein -cholesterol; TG: triglycerides).

Interestingly, benefits of CR are not only observed in obese subjects. Long-term (3–15 years) CR in non-obese humans has a profound beneficial impact on CV risk factors, such as serum total cholesterol, LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, TG, and BP [20]. A decrease in the inflammatory state is reflected by the extremely low levels of high-sensitivity C-reactive protein (CRP) detected in these subjects [20]. Since CV risk factors increase with age, CR reveals as a promising strategy to prevent the development of CVD in both obese and non-obese individuals. However, it has to be noted that long-term interventions might not be equivalent to short-term interventions in

the context of obesity and/or metabolic dysfunction, which may include increased resilience and/or improvements in indices of disease risk but are not associated with delayed aging (Figure 1).

3. CR Protocols Differ in Their Starting Point, Severity, Duration and Number of Phases

To assess and compare the effectiveness of CR protocols on CVD several aspects, such as CR severity, its duration, starting point and number of phases, need to be taken into account (Figure 2). A comparison of several approaches is shown in Table 1.

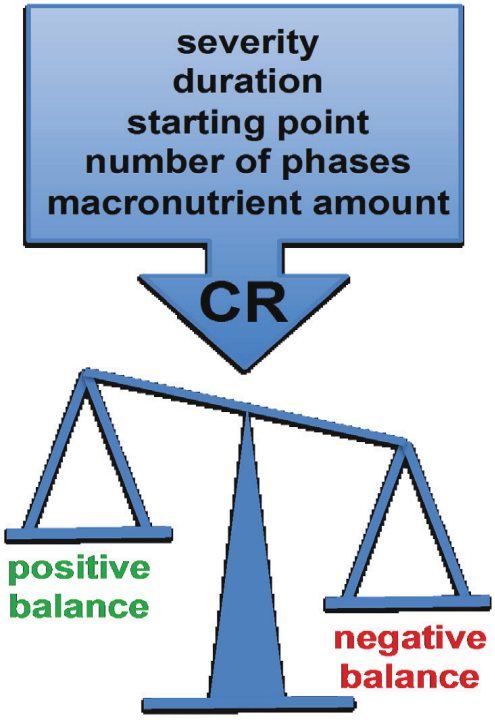

Figure 2. Critical aspects in a CR to achieve the desired effects. Although effects exerted by CR have been widely studied, there is no agreement in how a CR must be in order to prevent CV events. Numerous studies suggest that its severity, duration, starting point, number of phases and composition (*i.e.*, macronutrient amount) are important aspects to take into account. Modifying all these characteristics in different ways can exert a positive or a negative balance, thus affecting CV risk factors (CR: caloric restriction; CV: cardiovascular).

There is no agreement on how severe a CR must be in order to confer benefits in different organs and systems. In general, the *ad libitum* (AL) caloric intake is reduced around 20%–40% exerting positive effects without deleterious consequences [21]. However, numerous protocols include an alternate-day fasting, in which caloric intake reduction will be intermittent [22,23]. Most of the CR protocols reduce very intensively the energetic consumption for a long time. Some examples include a daily 30%–40% reduction for 12 months or longer [24,25]. Others use comparable approaches but only for four or five weeks, obtaining similar effects [26]. This suggests that a CR does not need to be prolonged for a long time to be effective, with the advantage that short-term CR is easier to include in clinical practice. In this context, a genomic analysis revealed that the results obtained after CR during four weeks were similar to those from longer CR (28 weeks). In this study, the short-term CR revealed the 70% of

the effects observed under the long-term CR on liver gene expression with age [27]. However, other authors report different findings in other tissues, such as in white adipose tissue [28], probably due to variations in responding to fasting cycles. Overall, we feel that most of the studies do not reflect a realistic intervention since they include really severe protocols.

Table 1. Comparison of several caloric restriction (CR) protocols in rodents and humans.

Reference	Model	CR Protocol
Kondo *et al.*, 2009 [26]	C57/BL6 mice	35% CR for 4 weeks
Donato *et al.*, 2013 [29]	mice	10% CR (1 week) + 25% CR (1 week) + 40% CR throughout the life of the animal
Chen *et al.*, 2015 [30]	Wistar rats	20% CR or 40% CR for 12 weeks—only reduction of starch
Chou *et al.*, 2010 [31]	Wistar rats	40% CR for 2 weeks
Dolinsky *et al.*, 2010 [32]	Wistar and SHR rats	10% CR (2 weeks) + 40% CR (3 weeks)
Chandrasekar *et al.*, 2001 [33]	Fisher344 rats	40% CR for 10 months
Csiszar *et al.*, 2009 [34]	Fisher344 rats	40% CR (life-long; age-related studies)
Ahmet *et al.*, 2011 [24]	Fisher344 rats	40% CR for 22 months (age-related studies)
Zanetti *et al.*, 2010 [35]	Fisher344 rats	26% CR for 3 weeks (age-related studies)
Castello *et al.*, 2005 [36]	Sprague Dawley rats	40% CR for 4, 10 or 22 months (age-related studies)
Ozbek *et al.*, 2013 [37]	Sprague Dawley rats	40% CR for 3 months
Minamiyama *et al.*, 2007 [38]	type II diabetic rats (OLETF)	30% CR for 13 weeks
García-Prieto *et al.*, 2015 [39]	Zucker obese rats	20% CR for 2 weeks
Ketonen *et al.*, 2010 [40]	C57Bl/6J mice under HFD	30% CR for 50 days (with HFD)
Iacobellis *et al.*, 2008 [41]	patients	VLCD (900 kcal/day). Phase 1—complete meal replacement (12 weeks); phase 2—transition period including healthy foods and partial meal replacement (4–6 weeks); phase 3—long-term maintenance
Kitada *et al.*, 2013 [19]	overweight patients	25% CR for 7 weeks
Siklova-Vitkova *et al.*, 2012 [42]	obese patients	800 kcal/day (1 month) + weight stabilization period (low-calorie diet for 2 months + weight maintenance diet for 3 months)
Capel *et al*, 2009 [43]	obese patients	800 kcal/day (1 month) + weight stabilization period (low-calorie diet for 2 months + weight maintenance diet for 3–4 months)
Davì *et al.*, 2002 [9]	obese patients	1200 kcal/day for 12 weeks
Ziccardi *et al.*, 2002 [10]	obese patients	1300 kcal/day for 12 months
Raitakari *et al.*, 2004 [11]	obese patients	580 kcal/day for 6 weeks
Cooper *et al.*, 2012 [15]	obese patients	CR to produce a 8%–10% weight loss within 12 months with or without physical activity
Morel *et al.*, 2011 [44]	obese patients	600 kcal/day (1 month) + 1200 kcal/day (1 month)
Fontana *et al.*, 2007 [13]	overweight/obese patients	16% CR (3 months) + 20% CR (9 months)
Ho *et al.*, 2015 [18]	overweight/obese patients	CR to produce a 5%–7% weight loss within 12 months
Murakami *et al.*, 2007 [45]	overweight/obese patients	≈1200 kcal/day (women) or 1600 kcal/day (men) for 12 weeks with or without exercise program
Sasaki *et al.*, 2002 [8]	obese patients with hypertension	800 kcal/day for 2 weeks

AL: *ad libitum;* CR: caloric restriction; HFD: high-fat diet; SHR: spontaneously hypertensive rats; VLCD: very low-calorie diet.

Initially, it was thought that benefits appeared only after long periods of CR [46]. Nevertheless some of the beneficial effects promoted by CR, such as plasmatic glucose levels decrease, show up within the first week of the diet [47]. Important protection of endothelial function occurs in vascular aging models even under CR protocols for less than three weeks [35,48]. Other vascular aged-related complications, such as aortic stiffening and artery wall hypertrophy, need longer dietary treatments to be prevented [29].

Regarding the starting point of a CR, its effects on lifespan are higher if it is initiated at the weaning [49–51]. A study in 10-week-old spontaneously hypertensive rats (SHR), with a moderate hypertension, showed that a 10% CR for two weeks followed by a 40% CR for three additional weeks avoided the increase in BP levels [32]. However, starting the same protocol at older ages, when BP values were higher (>200 mmHg) could not reduce those values. This suggests that CR protocols might be more beneficial at early stages of vascular disease [32].

The different phases in which a CR can be subdivided play a substantial role in the achievements can be reached. In multiphase dietary interventions, the pattern of adipokine expression, secretion rate, and plasma levels is different with respect to the phase of the intervention, and to the cellular origin of the respective adipokine [42]. Adipocyte-derived adipokines (adiponectin, leptin, serum amyloid A, or haptoglobin) decrease (except for adiponectin), during the initial very low-calorie diet (VLCD), whereas they increase toward prediet levels during the weight stabilization phase. Similar results have been observed with low-calorie diets [52]. In contrast, expression and secretion rate of stromal-vascular fraction-derived adipokines (tumor necrosis factor α TNF-α, interleukins IL-6, IL-10, IL-8, monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1, PAI-1) increased or remained unchanged during the initial VLCD but decreased during the weight stabilization phase [42]. During the various phases of a dietary weight loss program, adipose tissue macrophages and adipocytes show distinct patterns of gene regulation and association with insulin sensitivity, the regulation of gene expression being dependent on the severity and duration of CR [43].

In conclusion, the optimal CR protocol for each specific situation remains to be determined and standardization will be necessary to allow comparison of the beneficial effects of different dietary approaches on CVD.

4. Mechanisms by Which CR Exerts Vascular Protection in Metabolic Disorders

The improvement of vascular dysfunction associated with metabolic disorders might be due to changes in endothelial function, in the arterial wall structure, or in the paracrine effects of perivascular adipose tissue (PVAT).

4.1. Effects of CR on Endothelial Function

CR leads to an improvement of endothelial function in arteries from several models of aged [24,29,33,34,36,53] and obese rodents [38–40]. Underlying mechanisms include the increase of nitric oxide (NO) bioavailability [29,31,35,48] due to the enhancement of endothelial nitric oxide synthase (eNOS) expression and/or activity [29,37,54], together with the suppression of vascular oxidative stress associated to superoxide anion (O_2^-) production [29,31,34,38,40] and lipid peroxidation [38]. Vasoprotective effects of life-long CR both reduce the expression and activity of NADPH oxidase and O_2^- production, and enhance superoxide dismutase (SOD) activity [29].

Endothelial function improvement is also achieved by the reduction of inflammatory mediators, such as CRP, which is a moderately good predictor of acute vascular events related to obesity [55], IL-6 and TNF-α in mice [55], as well as decreases in nuclear factor NF-κB activity [33,34].

A key event associated with the improvement of endothelial function under CR is the activation of the AMPK-PI3K-Akt-eNOS signaling pathway. A dysregulation of this pathway in over-nutrition and obesity contributes to the development of endothelial dysfunction (for review, see [56]) in both diet-induced [57] and genetic models of obesity, such as Zucker *fa/fa* obese rats [58], Zucker diabetic fatty rats [59] or Otsuka Long Evans Tokushima Fatty rats [60]. CR triggers endothelial AMPK activation leading to (i) normalization of endothelial function and systolic BP reduction in Zucker obese rats [39]; (ii) an improvement of vascular compliance and BP reduction in hypertensive rats [32]; or (iii) revascularization in response to ischemia [26]. Moreover, activation of AMPK by CR reduces lipotoxicity associated with high-fat diets (HFD), insulin resistance, and obesity by decreasing the excessive exposure of the endothelium to free fatty-acids (FFAs) [61,62], thus exerting a protective effect on endothelial cells [61,62]. Obese patients subjected to CR that reported systolic BP reduction, as

well as FFA and inflammatory marker level decrease, showed an increase of AMPK phosphorylation in peripheral blood mononuclear cells [19]. The fact that these changes are observed even with a moderate reduction in BW supports the predominant role of the negative energy balance to achieve CV benefits of CR.

Activation of the cellular sensing protein sirtuin-1 (SIRT1) is another mechanism proposed as key mediator of vascular benefits of CR [29,34]. Arterial aging is associated with changes in expression and/or activity of SIRT1 and mammalian target of rapamycin (mTOR), which are ameliorated by life-long CR. In the same line, serum obtained from CR patients activates SIRT1 in human cells *in vitro* [63]. There are, however, very few studies assessing the vascular role of SIRT1 activation by CR in obesity, and further research needs to be performed in this line.

4.2. Effects of CR on Arterial Wall Structure and Remodeling

Subclinical organ damage, such as vascular remodeling, precedes the occurrence of CV events in individuals with obesity and hypertension [64]. Life-long CR significantly reduces large elastic artery wall hypertrophy and prevents aortic stiffening in mice [29] due to a suppression of collagen production [29] and elastin fiber degradation [53]. Increases in elasticity after CR have also been described in arteries from young, but not from aged SHR [32]. This suggests that reversal of early changes in the arterial wall structure, at a time when vascular remodeling is emerging and still reversible, is essential for the prevention of vascular dysfunction.

Mechanisms underlying CR-induced changes in vascular remodeling have been mainly analyzed in models of aging. CR introduced early in life protects against aortic fibrosclerosis by decreasing oxidative damage and consequently reducing the levels of transforming growth factor beta-1 (TGFβ1). This change very likely occurs through a downregulation of the mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK) and activator protein-1 (AP-1) signaling pathways [36]. Whether the same mechanisms underlie the effects of CR on metabolic arterial wall remodeling remains to be further analyzed.

Both intervention and cross-sectional studies in humans demonstrate that short-term CR and reduced BW are associated with lower BP and carotid wall thickness [15,16,20,65]. A weight loss of around 8% results in a mean decrease of 0.07 mm in carotid artery diameter, whereas individuals who achieved at least a 5% weight loss showed a significant reduction in mean carotid intima:media thickness [15]. A meta-analysis of 43 studies on moderate weight loss (around 11%) due to CR, with or without exercise, demonstrates an improvement of some arterial compliance and stiffness parameters, such as cardio-ankle vascular and β-stiffness index, arterial compliance and distensibility, distal oscillatory compliance, proximal capacitive compliance, systemic arterial compliance or reflection time. However, other parameters, such as augmentation index, strain, augmentation pressure and pulse pressure were not significantly improved with weight loss [66]. All of these results demonstrate that an intensive dietary intervention at a time when vascular remodeling has only been initiated and is not irreversibly established might significantly reverse some of the key adverse vascular structural changes associated with excess weight in severely obese adults.

4.3. Effects of CR on PVAT Dysfunction in Obesity

Perivascular adipose tissue (PVAT) is the adipose tissue surrounding most blood vessels, except cerebral vessels. It might be white or brown adipose tissue and produces a number of vasoactive factors, adipokines and cytokines. A large body of evidence supports the paracrine influence of PVAT for the maintenance of vascular resistance under physiological and pathophysiological conditions [67]. PVAT releases a number of adipokines, inflammatory cytokines, and other vasoactive factors, which are variable in quantity and pattern depending on the PVAT amount [67]. In fact, obesity triggers an increase in PVAT throughout the vasculature [68,69], accompanied by an unbalance in favor of vasoconstrictor and pro-inflammatory substances, which leads to endothelial dysfunction and vascular damage [70–72]. Long-term HFD in mice leads to PVAT dysfunction, characterized by an increase in

NADPH oxidase activity and O_2^- release, as well as by a reduction in extracellular SOD expression, total SOD activity, eNOS levels, and NO availability [70]. Similarly, PVAT of New Zealand obese mice show increased O_2^- levels formation and decreased SOD expression, leading to an impaired hydrogen peroxide (H_2O_2) production, which contributes to vascular dysfunction reducing the anti-contractile effects of PVAT [73]. In Ossabaw obese swine H_2O_2-mediated vasodilatation was markedly attenuated by the presence of coronary PVAT [74]. Ma *et al*. [72] showed that PVAT induces endothelial dysfunction by dysregulation of the AMPK/mTOR pathway in the aorta of diet-induced obese rats, characterized by a downregulation of AMPK-eNOS pathway and a concurrent upregulation of mTOR. Moreover, obese periaortic PVAT high FFA levels could attenuate the anti-contractile properties of PVAT by a TNF-α-dependent [75].

An interesting issue, which deserves future investigation, is the impact of the diet composition on oxidative stress in PVAT. A fructose-rich diet, which decreases polyunsaturated FAs and increases saturated and monounsaturated FAs in PVAT impairs vascular function by a decrease in antioxidant enzymes and a reduction in glutathione content [76]. Altogether, these results indicate that obesity and the diet composition induce a switch in PVAT to a more pro-inflammatory, pro-oxidant and vasoconstrictor phenotype.

Since the beneficial or deleterious paracrine influence of PVAT is directly dependent on its amount [77,78], a key question is whether the proportion of adipose tissue loss induced by CR is uniform (overall adiposity) or predominant in specific adipose depots (*i.e.*, PVAT). Significant reduction in the thickness of epicardial fat (surrounding coronary arteries) has been described both in severely obese patients who underwent substantial weight loss after bariatric surgery [79], as well as after a short-term VLCD program [41]. Interestingly, the decrease of epicardial fat is substantially higher than changes in overall BW loss body mass index and waist circumference, and correlates with the improvement in both left ventricular mass and diastolic function. Interestingly, bariatric surgery also reverses the obesity-induced damage to PVAT anticontractile function by reducing adipocyte hypertrophy, PVAT inflammation and increasing both PVAT-derived NO and adiponectin availability [80]. We believe that these studies open a new approach for the management of vascular damage and CV risk associated with PVAT dysfunction in metabolic related disorders.

4.4. Effects of CR on Vascular Actions of Leptin and Adiponectin

Obesity is associated with hyperleptinemia and hypoadiponectinemia, both playing a key role in the pathogenesis of endothelial dysfunction [81]. Chronic hyperleptinemia has been linked to endothelial dysfunction and damage [82–84] stimulating the increase in NADPH oxidase activity and O_2^- production in the vascular wall, as well as promoting glutathione peroxidase activation to remove excessive H_2O_2 production. On the other hand, hypoadiponectinemia is closely associated with endothelial dysfunction in humans and adiponectin knock-out mice show a decrease in eNOS phosphorilation levels.

Different CR protocols in rats, markedly changes the adipokine production pattern leading to an increase in circulating levels of adiponectin, whereas leptin levels profoundly decrease in adipose tissue [85,86].

A role for adipokine levels in PVAT and their paracrine influence on the vascular wall might not be discarded. *Ob*/*ob* mice lacking leptin do not exhibit NO production in perivascular adipocytes. Interestingly, this is restored in PVAT after two-week subcutaneous leptin infusion, suggesting that NO release in PVAT seems to be mediated by leptin [87]. Hypoadiponectinemia in patients with T2D stimulates vascular NADPH oxidase expression, which is counterbalanced by upregulation of adiponectin expression in PVAT aimed at suppressing in a paracrine manner NADPH oxidase activity via a PI3K/Akt-mediated deactivation of Rac1 and the downregulation of $p22^{phox}$ gene expression [88]. These studies again stress the concept that PVAT-vessel interaction is a promising therapeutic target for the prevention of vascular complications of metabolic disorders. The effect of CR and dietary approaches on this interaction deserves future investigation.

5. Dietary Strategies Based on Macronutrients Modification

Not only the amount but also the quality of the nutrient intake contributes to benefits on vascular function [89–91]. However, any dietary change can be universally applied as positive at the CV level since their beneficial effects are different depending on the metabolic state of the individual. Although dietary interventions need to be personalized on the basis of patient requirements, some of the most widely approaches used in clinical practice regarding macronutrients content on the diet and its effects at vascular level are described in Sections 5.1 to 5.3 below.

5.1. Low-Carbohydrate Diets

In both overweight and obese patients, carbohydrate restricted diets are more effective than low-fat diets in terms of atherogenic dyslipemia and other metabolic syndrome characteristics such as inflammation, oxidative stress and CV risk markers (*i.e.*, apolipoproteins A-1 and B, LDL particle distribution, and FMD) [92–95]. Peripheral small artery vascular function is improved with low carbohydrate diets when there is a decrease in BW [96,97]. However, some authors report that the degree of weight loss in obese patients is not correlated to changes in BP, endothelial function and inflammatory markers [98]. Restricting dietary carbohydrate positively impacts lipid homeostasis and inflammatory markers (*i.e.*, TNF-α, IL-6, PAI-1) even when the saturated fat intake is higher due to the isocaloric replacement of carbohydrates [92–95]. Dysregulation of PAI-1 is involved in enhanced inflammation, vascular damage and subsequent thrombogenicity [99]. Thus, studies with different dietary interventions aim to establish PAI-1 as a possible therapeutic target for controlling CVD in obese patients [44,45]. Moreover, beneficial effects of carbohydrate restriction in combination with pharmacological treatments, *i.e.*, statins, have been described [98]. A decrease in approximately 400 kcal/day for six weeks (with carbohydrates representing the 11% of the daily intake) reduced CV risk markers such as serum TG, soluble E-selectin and intracellular adhesion molecule-1. Both systolic and diastolic BP decreased together with an increase in reactive hyperemia, indicating a better resistance vessel endothelial function, probably in a prostaglandins-mediated way and independent of NO increase [98,100].

However, there is no consensus regarding how much the carbohydrate amount of the diet must be reduced to be beneficial. A recent study comparing the effects of two low-carbohydrate diets for 12 weeks in rats (80% energy intake with 34% carbohydrate reduction *vs.* 60% energy intake with 68% carbohydrate reduction of the control diet) demonstrated that decreasing carbohydrate intake to a large extent even with a lower caloric intake has detrimental effects on lipid balance due to an increase in LDL and total cholesterol levels [30]. Indeed, dietary patterns characterized by a low amount of carbohydrate but reciprocally higher content in fat and protein are related to a poorer vascular reactivity in overweight and obese patients [97] and in patients with T2D and increased vascular risk [101]. This emphasizes the necessity of studying the impact of macronutrient composition and nature on CVD (Figure 3).

5.2. Higher Protein Content: Does It Make the Difference?

Diets low in carbohydrates have been proven to reduce BW in both overweight and obese patients [91,96,97,102] and to maintain weight when associated with a moderate increase in protein amount [103]. Low-fat/high-protein diets are more effective for long-term maintenance or further improvement of vascular function [104,105]. Thus, energy-restricted diets are generally compensated by protein content, usually with meal replacements [101,105]. Obese T2D subjects receiving either a meal replacement-based low-calorie diet (approximately 1000 kcal/day) or a low-fat, high-protein, reduced-carbohydrate diet (HP, intake decrease about 600 kcal/day) for eight weeks and either switching to or continuing with the HP diet for 44 additional weeks showed similar positive impacts on endothelial function and inflammatory markers [105]. Both diets reduced blood glucose, TG and LDL-cholesterol and improved endothelial function by lessening soluble E-selectin and increasing

FMD. However, only the HP diet decreased CRP and IL-6 levels, markers of inflammation related to vascular homeostasis. Controlled trials in which obese patients were assigned to two periods of a four-week hypocaloric diet (either high in proteins or a conventional one, 1200 kcal/day) [106] showed similar results in some metabolic risk markers after both diets. Interestingly, diets high in protein were associated with improvement in some cardiometabolic risk factors such as PAI-1, vascular endothelial growth factor, fasting plasma glucose and CRP despite the fact of its higher fat content. However, only the conventional hypocaloric diet decreased systolic BP, highlighting the importance of every macronutrient maintaining vascular homeostasis in obese patients [106]. Since high CRP precedes atheromatic events [107], lessening its levels could pave the way to better prevent CV disease. Some clinical trials (*i.e.*, the OmniHeart study) support the idea that a partial replacement of carbohydrates with either protein or monounsaturated fat has additional beneficial effects on the basis of a healthy diet at a vascular level independent of BW loss in both non-obese and obese patients with prehypertension or stage 1 hypertension [108]. Without reducing caloric intake (2100 kcal/day), these patients further reduced systolic and diastolic BP and decreased estimated risk of suffering coronary heart disease with both diets rich in protein or rich in unsaturated fat [108].

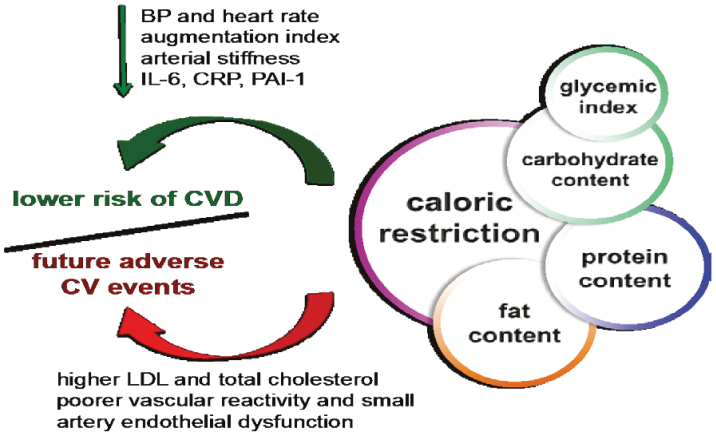

Figure 3. CV outcome of CR depends on macronutrient composition of the diet. Both the amount and the quality of each macronutrient of the diet are important in order to achieve positive effects at CV level. A proper balance in macronutrient amount is key to avoid future adverse CV events (BP: blood pressure; CR: caloric restriction; CRP: high-sensitivity C-reactive protein; CV: cardiovascular; CVD: cardiovascular disease; IL-6: interleukin 6; LDL: low-density lipoprotein; PAI-1: plasminogen activator inhibitor-1).

Besides the positive effects that a protein supplementation can exert, replacing carbohydrates with higher amounts of protein and fat lead to a low small artery reactive hyperaemia index, a marker of small artery endothelial function [101], which correlates with endothelial dysfunction [109], thus predicting future adverse CV events [110]. Hence, balancing the amount of each macronutrient of the diet might be of importance for achieving positive effects at the CV level (Figure 3).

5.3. Diet Glycemic Index Variation

Both the carbohydrate and fat content of a diet influences the occurrence of diabetes and CVD [102,111]. Although diets restricting carbohydrate consumption are a well-known strategy for weight loss with the subsequent improvement of metabolic-related complications in overweight and obese patients [102], recent studies show that following these diets for a long time does not always protect from CVD [101,112,113]. In this context, changing carbohydrate quality rather than

quantity has emerged as a better approach to dealing with CV risk factors. Glycemic index (GI) is the quantification of the blood glucose response to a carbohydrate compared to a carbohydrate reference [114]. That is, foods with similar carbohydrate content can behave differently in terms of raising blood glucose. Thus, varying GI might be useful in the management of these diseases (Figure 3). The effects on metabolism exerted by different GI diets are diverse [89,91,115]. Control trials in which obese patients at increased CV risk were subjected to hypocaloric diets with different GI for three months showed a decrease in BW, plasma glucose, total cholesterol, LDL-cholesterol, TG, systolic BP and heart rate independent of the GI of the diet [91]. Interestingly, FMD was influenced, being higher after following the low GI diet [91], a fact observed in studies assessing the acute effects of different GI diets [89]. In this context, the EVIDENT study (2010, Spain) aims to establish an association between GI and vascular homeostasis by assessing arterial stiffness (via pulse wave velocity measurement) and augmentation index (vascular aging related to endothelial dysfunction) [116]. Their latest results show that an increase in GI correlates with an enhancement of augmentation index, thus increasing the risk of suffering a CVD [116]. Low GI diets can reduce oxidative stress [117] and inflammation [90], factors widely associated with endothelial dysfunction. In this context, low GI hypocaloric diets have been shown to decrease CRP blood levels in overweight patients [90]. However, some clinical trials claim that GI is not related to an improvement in CV risk factors on the basis of a healthy diet [118]. In this controlled feeding study, the effects of four different interventions on carbohydrate content from a healthy diet without losing weight were analyzed. No differences were observed between high and low GI diets regarding insulin sensitivity, lipid homeostasis and systolic BP [118]. All of these diverse results between studies may be due to changes in content of total carbohydrates and fibers, no concomitant weight loss and duration of the dietary treatment, although long-term studies with BW decrease conclude in a similar way [90,119].

6. Conclusions

All the described dietary modifications are effective by mitigating CV risk factors in obesity. However, despite all the efforts made to establish CR or a balanced diet as the main strategies to either prevent or treat CV complications related to metabolic disorders, there is no fully effective formula, and factors such as severity, duration, starting point, number of phases and composition need to be taken into account. It is important to note that an intensive dietary intervention at a time when vascular dysfunction has only been initiated and is not irreversibly established might significantly reverse some of the key adverse vascular changes associated with obesity.

On the other hand, many mechanisms underlying CR-induced changes in vascular dysfunction have been mainly analyzed in models of aging (*i.e.*, vascular remodeling). Whether the same mechanisms underlie the effects of CR on obesity related vascular dysfunction remains to be further analyzed. In addition, most of these regimens have the ability to attenuate some, but not all, of the components involved in this complicated multifactorial condition. Therefore, further research is needed to understand in depth the mechanisms involved in the positive effects of CR and macronutrient intake modification on vascular dysfunction associated with being overweight and obese.

Moreover, we need to define specific biomarkers to (i) assess and/or predict the effect CR on CV risk and (ii) design an optimal and more personalized dietary regime, taking into account individual differences in age, metabolic function and CV risk factors.

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Abbreviations

The following abbreviations are used in this manuscript:

Akt	protein kinase B
AL	*ad libitum*
AMPK	adenosine monophosphate-activated protein kinase
AP-1	activator protein-1
BP	blood pressure
BW	body weight
CR	caloric restriction
CRP	high-sensitivity C-reactive protein
CV	cardiovascular
CVD	cardiovascular disease
eNOS	endothelial nitric oxide synthase
FA	fatty acid
FFAs	free fatty-acids
FMD	flow-mediated dilation
GI	glycemic index
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HFD	high-fat diet
HP	low-fat, high-protein, reduced-carbohydrate
IL	interleukin
JNK	c-Jun N-terminal kinase
LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein-1
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor κB
NO	nitric oxide
O_2^-	superoxide anion
PAI-1	plasminogen activator inhibitor-1
PI3K	phosphoinositide 3-kinase
PVAT	perivascular adipose tissue
SHR	spontaneously hypertensive rats
SIRT1	sirtuin-1
SOD	superoxide dismutase
T2D	type 2 diabetes
TG	triglycerides
TGFβ1	transforming growth factor beta-1
TNF-α	tumor necrosis factor α
VLCD	very low-calorie diet

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Review

NO-Rich Diet for Lifestyle-Related Diseases

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Abstract: Decreased nitric oxide (NO) availability due to obesity and endothelial dysfunction might be causally related to the development of lifestyle-related diseases such as insulin resistance, ischemic heart disease, and hypertension. In such situations, instead of impaired NO synthase (NOS)-dependent NO generation, the entero-salivary nitrate-nitrite-NO pathway might serve as a backup system for NO generation by transmitting NO activities in the various molecular forms including NO and protein *S*-nitrosothiols. Recently accumulated evidence has demonstrated that dietary intake of fruits and vegetables rich in nitrate/nitrite is an inexpensive and easily-practicable way to prevent insulin resistance and vascular endothelial dysfunction by increasing the NO availability; a NO-rich diet may also prevent other lifestyle-related diseases, including osteoporosis, chronic obstructive pulmonary disease (COPD), and cancer. This review provides an overview of our current knowledge of NO generation through the entero-salivary pathway and discusses its safety and preventive effects on lifestyle-related diseases.

Keywords: lifestyle-related disease; nitric oxide (NO); nitrate; nitrite; insulin resistance; ischemia/reperfusion injury; chronic obstructive pulmonary disease (COPD); osteoporosis; cancer

1. Introduction

Health problems, such as insulin resistance, cardiovascular disease, osteoporosis, and cancer share some common risk factors, including unhealthy and excessive nutrition, a lack of physical activity, smoking and heavy drinking [1]. So-called lifestyle-related diseases are now the leading causes of mortality and morbidity in developed countries [2]. Healthy diets and exercise training are low-cost and easily-practicable lifestyle changes to be recommended for patients with these conditions before starting pharmacological therapy. Recent prospective and epidemiologic studies have shown that among the various foods, green leafy vegetables are undoubtedly protective against coronary heart disease, hypertension [3–6], and ischemic stroke [7]. This may be because vegetables and fruits rich in nitrate can provide a physiological substrate for reduction to form nitrite and nitric oxide (NO). The beneficial effects of these foods on the diseases resulting from circulatory disturbances are attributed to the cyclic guanosine monophosphate (cGMP)-dependent actions of NO, including vasodilation and vascular endothelial protection from platelet aggregation and leukocyte adhesion [8]. However, in addition to these classical functions of NO, recent studies have indicated novel functions for NO through cGMP-independent and protein *S*-nitrosylation-dependent intracellular signaling pathways [9]. *S*-nitrosylation is associated with the activation of transcription factors, the regulation of a number of signal transduction molecules [10] and redox protein modification [11], mitochondrial functions [12,13], and cell apoptosis [14], which could explain how the dietary nitrate exerts preventive effects against the development of lifestyle-related metabolic, inflammatory, and proliferative disorders. This review provides an overview of our current knowledge of NO production through the dietary nitrate-nitrite-NO pathway and its physiological aspect, then discusses the safety and efficacy of dietary nitrate, as well as its preventive effects on lifestyle-related diseases.

2. The Dietary Nitrate-nitrite-NO Pathway and Its Physiological Aspect

In addition to endogenous NO generation through the L-arginine-NO synthase (NOS) pathway, NO is also generated through the NOS-independent nitrate-nitrite-NO pathway [15] (Figure 1). Green leafy vegetables such as lettuce, spinach and beetroot all contain high concentrations of nitrate [16] (Table 1). Vegetables account for 60%–80% of the daily nitrate intake in a Western diet [17], and substantial elevations in the plasma nitrite levels can occur through increasing the dietary nitrate intake [18]. One serving of such a vegetable contains more nitrate than what is endogenously generated by NOS isoforms during a full day in humans [19]. As shown in Figure 2, the ingested dietary nitrate is absorbed in the upper gastrointestinal tract and reaches the peak plasma level around 30–60 min after ingestion [20] (Table 2). Approximately 25% of the absorbed nitrate is delivered to the salivary gland and secreted into saliva in the oral cavity [21], which is then reduced to nitrite by commensal anaerobic bacteria on the tongue [22]. In the acidic environment of the stomach, part of the swallowed nitrite is immediately protonated to nitrous acid ($NO_2^- + H^+ \rightarrow HNO_2$), then decomposed to form a variety of nitrogen oxides such as NO, nitrogen dioxides (NO_2), and dinitrogen trioxide (N_2O_3) ($2\ HNO_2 \rightarrow N_2O_3 + H_2O$, $N_2O_3 \rightarrow NO + NO_2$) [23]. These nitrogen oxides form additional bioactive adducts, such as *S*-nitrosothiols and *N*-nitrosoamines, following reactions with protein thiols and amines, respectively, in the dietary products. In particular, the NO production in the stomach is greatly enhanced in the presence of dietary polyphenols [24] and ascorbic acid [15], whereas because of its lower stability and shorter half-life relative to *S*-nitrosothiols, the released NO in the stomach is thought to locally contribute to increasing the gastric mucosal blood flow and mucous thickness to ensure the normal gastric physiology, and serves as the first-line host defense against swallowed pathogens [25,26]. However, some of the nitrite escapes the protonation in the acidic milieu of the stomach and enters the systemic circulation, and then reaches the peripheral organs, including skeletal muscles, where it acts in an endocrine manner to exert NO-like activity [18] (Figure 2). Because the plasma levels of nitrite are highly dependent on the amount of salivary nitrate and its reduction to nitrite, the use of an antibacterial mouthwash [27] and frequent spitting of saliva consequently decrease the plasma levels of nitrite [20].

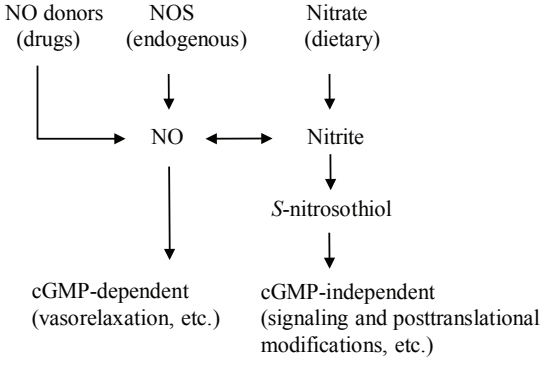

Figure 1. The NO pathways. NO is originated from the diet, drugs, and the endogenous NOS enzyme. The activities of NO are exerted through cGMP-dependent and independent ways. NO donor drugs, such as nitroglycerin and sodium nitroprusside, directly release NO. NOS endogenously generates NO, which is linked to the cGMP-dependent functions of NO. Dietary nitrate/nitrite produce NO and *S*-nitrosothiol, mainly for cGMP-independent functions. NO: nitric oxide, NOS: NO synthase, cGMP: cyclic guanosine monophosphate.

Table 1. Nitrate and nitrite contents of food products.

Food product	Nitrate concentration (mg/100 g)		Nitrite concentration (mg/100 g)		Reference
	Mean	Range	Mean	Range	
Beets	275.6	168–359	1.00	0.21–2.98	[16]
Spinach	233.3	53.5–366	0.70	0.0–1.29	
Radishes	168.0	76.4–250	0.01	0.0–0.1	
Celery	154.4	31.6–332	0.16	0.0–0.52	
Lettuce	85.0	7.9–217.1	0.06	0.001–0.97	
Iceberg lettuce	78.6	34.7–108	0.02	0.0–0.17	
Mushroom	59.0	1.9–8.5	0.80	0.0–3.8	
Cabbage	57.3	19.3–97.6	0.24	0.0–1.26	
Broccoli	39.4	2.9–114	0.06	0.001–0.95	
Green beans	38.6	16.5–61.1	0.05	0.0–0.25	
Strawberries	17.3	10.5–29.3	0.20	0.0–0.71	
Banana	13.7	8.8–21.4	0.21	0.0–0.95	
Green pepper	3.3	0.8–5.5	0.04	0.0–0.3	
Spinach	741.0	-	0.02	-	[28]
Mustard greens	116.0	-	0.003	-	
Salad mix	82.1	-	0.13	-	
Cole slaw	55.9	-	0.07	-	
Broccoli	39.5	-	0.07	-	
Tomato	39.2	-	0.03	-	
Vegetable soup	20.9	-	0.001	-	
Hot dog	9.0	-	0.05	-	
Bacon	5.5	-	0.38	-	
Banana	4.5	-	0.009	-	
Pork tenderloin	3.3	-	0.0	-	
Bacon nitrite-free	3.0	-	0.68	-	
French fries	2.0	-	0.17	-	
Ham	0.9	-	0.89	-	
Fruit mix	0.9	-	0.08	-	
Orange	0.8	-	0.02	-	
Apple sauce	0.3	-	0.008	-	
Ketchup	0.1	-	0.13	-	
Carrots	0.1	-	0.006	-	
	Nitrate concentration (mg/L)		**Nitrite concentration (mg/L)**		
Carrot juice	27.55	-	0.036	-	
Vegetable juice *	26.17	-	0.092	-	
Pomegranate juice	12.93	-	0.069	-	
Cranberry juice	9.12	-	0.145	-	
Acai juice	0.56	-	0.013	-	
Green tea	0.23	-	0.007	-	

* V8; Campbell Soup Co (Camden, NJ, USA); Table is reproduced from the reference [16] and [28].

Table 2. Saliva and plasma levels of nitrite, nitrate, and *S*-nitrosothiol before and 30 min after oral administration of sodium nitrate (10 mg/kg) in healthy volunteers.

		0 Min	30 Min
Saliva	Nitrite (μM)	104 ± 21	713 ± 150
	Nitrate (mM)	0.19 ± 0.03	8.2 ± 1
	S-NO (nM)	25 ± 9.8	297
Plasma	Nitrite (μM)	123 ± 19	229 ± 46
	Nitrate (mM)	30 ± 4	432 ± 44
	S-NO (nM)	6.3 ± 1.4	No significant change

Data from reference 20, *S*-NO: *S*-nitrosothiol.

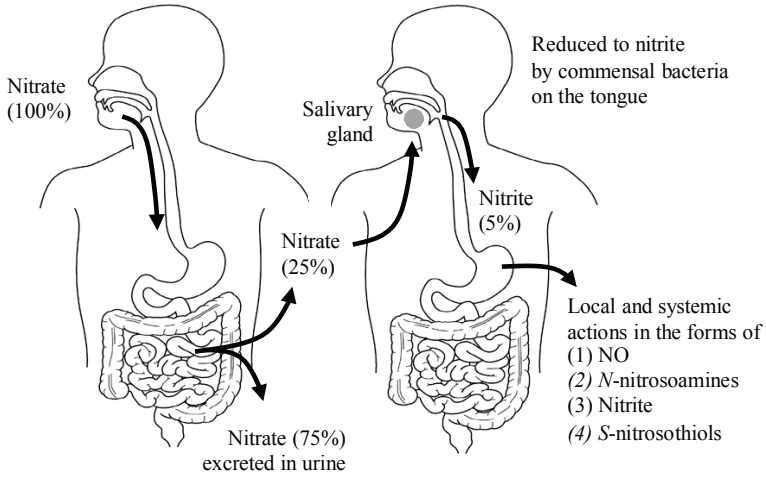

Figure 2. The entero-salivary nitrate-nitrite-NO pathway. Twenty-five percent of the ingested dietary nitrate is recycled to the saliva, and 20% of the nitrate in saliva is converted to nitrite by oral commensal bacteria. Approximately 5% of the originally ingested nitrate is swallowed into the stomach, and provides for NO activities in various forms. (1) NO for local vasodilation, mucus formation, and antimicrobial activity; (2) *N*-nitrosoamines for local carcinogenesis; (3) Nitrite for nitrite pool and transnitrosylation in the peripheral tissues; (4) *S*-nitrosothiols for transnitrosylation in the peripheral tissues.

The plasma nitrite which reaches peripheral tissues is stored in various organs. Although there have been few reports dealing with the tissue levels of nitrate/nitrite following dietary nitrate supplementation in humans, animal studies show that dietary nitrate certainly increases the tissue levels of nitrate/nitrite following increase in the plasma levels of nitrate/nitrite (Table 3), which accordingly exerts therapeutic efficacy for animal models of various disease conditions. Interestingly, while acute dietary nitrate intake increases the plasma levels of nitrite in rodents and humans [10,20], chronic dietary nitrate intake does not always increase the plasma and tissue levels of nitrite, but increases the tissue levels of nitrate and *S*-nitrosylated products (Table 3). Although the mechanism underlying this finding is yet to be clarified, there might be some redox equilibrium of nitrate-nitrite-NO after chronic dietary nitrate intake, resulting in oxidation or reduction of the tissue nitrite to form nitrate or *S*-nitrosylated species, respectively. On the other hand, animal models chronically fed a diet deficient in nitrate/nitrite exhibit significantly diminished plasma and tissue levels of nitrate/nitrite, resulting in increased ischemia-reperfusion injuries in heart and liver compared with the animal models fed a normal diet [29,30]. These results suggest that dietary nitrate intake is important in the maintenance of steady-state tissue levels of nitrate/nitrite for NO-mediated cytoprotection.

Various enzyme/protein-dependent reductions to NO have been proposed under physiological and pathological conditions, which include deoxyhemoglobin [31], deoxymyoglobin in the skeletal, vascular [32], and cardiac muscles [33], xanthine oxidase in endothelial cells, aldehyde oxidase, aldehyde dehydrogenase 2 [34], cytochrome P-450, and mitochondrial nitrite reductases (such as mitochondrial electron transport complexes) in all cells. In contrast to NOS-dependent NO production, which requires molecular oxygen, this nitrite reduction to NO is enhanced under hypoxic and acidic conditions. Because the nitrite-reducing factors are rich in skeletal muscles, tissue hypoxia during submaximal exercise kinetically favors nitrite reduction to NO, thus providing an alternative NO source for vascular dilation and efficient O_2 consumption in the working muscles [35,36].

Table 3. The effects of chronic dietary nitrate supplementation on tissue levels of nitrate/nitrite and therapeutic efficacy for experimental animal models.

Animal Model	Dietary Nitrate	Tissues	Effects of Dietary Nitrate	References
Uninephrectomized hypertension rat with high-salt diet.	Diets with 0.1 mM and 1 mM nitrate/kg/day for 8–11 weeks.	Kidney Heart Liver	Increase in plasma and tissue levels of nitrate and tissue levels of nitrosylation products. Reduction of oxidative stress and attenuation of renal injury, hypertension, cardiac hypertrophy and fibrosis.	[37]
C57BLK6 male mice with hypoxia-induced pulmonary hypertension.	0.6 mM, 15 mM, and 45 mM nitrate/L in drinking water for 3 weeks.	Lung	Increase in plasma and lung levels of nitrite and cGMP. Reduction of right ventricular pressure and hypertrophy, and pulmonary vascular remodeling.	[38]
Male Wistar rat with hypoxic heart damage.	0.7 mM/L nitrate in drinking water for 2 weeks.	Heart	Increase in plasma levels of nitrate and tissue levels of nitrite. Alleviation of metabolic abnormalities in the hypoxic heart. Improvement of myocardial energetics.	[39]

Although elevated plasma levels of nitrite certainly affect the cGMP production in systemic organs, providing an important signaling role in mammalian biology [10], dietary nitrate/nitrite also transmit biological signals via cGMP-independent mechanisms, such as transnitrosylation, a posttranslational modification analogous to phosphorylation, in order to regulate the protein function [40] (Figures 1 and 1). Here, the questions are raised. What is the carrier of NO activity to the peripheral organs, *S*-nitrosothiol, NO itself, or nitrite, and also, how does this carrier transnitrosylate in the peripheral organs? Lundberg *et al.*, showed that oral intake of sodium nitrate (10 mg/kg) in healthy volunteers significantly increased plasma levels of nitrite, but did not increase *S*-nitrosothiol in plasma [20] (Table 2). In addition, Bryan *et al.*, showed that protein *S*-nitrosylation in organs following intraperitoneal nitrite injection to rat, could not be inhibited by NO scavenging with carboxy-2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide (cPTIO), suggesting that the nitrite-mediated transnitrosylation in the organs might occur mainly directly through nitrite rather than through either circulating *S*-nitrosothiol or NO itself [10].

Bryan *et al.*, also showed that the increase in plasma nitrite within the physiological concentration rage of 0.2–2 μM after nitrite administration (plasma concentration of nitrite far lower than those required for vasodilation) enhanced *S*-nitrosothiol in the organs (heart, kidney, liver, lung, and aorta) with the subsequent modulation of signaling and gene expressions of cGMP, cytochrome P-450, heat shock protein 70, and heme oxygenase-1 in these organs. Interestingly, they also showed that a switch of the standard chow to the low nitrate/nitrite diet for two days in rats decreased nitrite levels substantially in all tissues and represented changes of the signaling and gene expressions in a direction opposite to those found with nitrite administration [10].

These observations indicate that the nitrite-induced transnitrosylation in organs might be an alternative *in vivo* nitrite signaling for the mammalian biology including protection of protein thiols from irreversible oxidation, transcriptional modulation, and posttranslational regulation of most classes of proteins present in all cells [9] (Figure 3), and also that changes in plasma nitrite levels even within the physiological ranges (e.g., postprandial and fasting) can affect tissue levels of *S*-nitrosothiol and subsequent cellular biology.

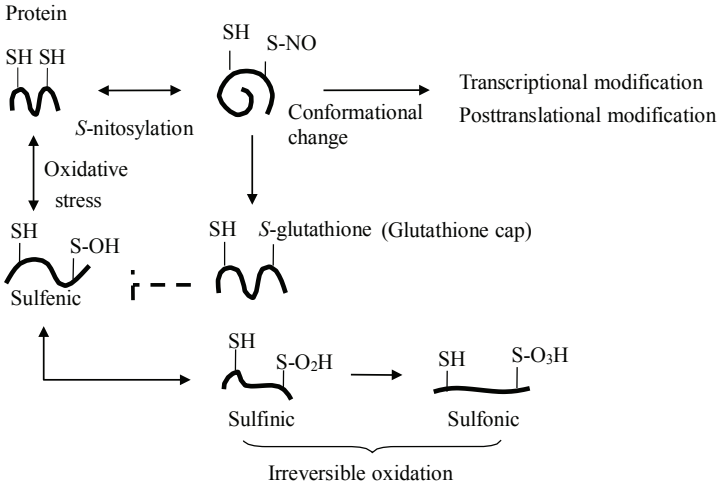

Figure 3. Protein *S*-nitrosylation. *S*-nitrosylation of protein elicits its regulatory effect by adding the NO moiety on the active thiol (SH of cysteine residue) of the protein (e.g., transcriptional factors and enzymes), and cell protection by the subsequent posttranslational addition of glutathione to the protein thiols (so-called glutathione cap), which shields the cysteine residues from further irreversible protein oxidation [11].

3. Safety and Efficacy of Dietary Nitrate

Very high concentrations of nitrate in drinking water may cause methemoglobinemia in infants (blue baby syndrome) [41]. In the 1940s, Comly first reported cases of cyanotic infants who received formula prepared with well water containing a high nitrate content [42]. Based on the subsequent analyses of the infantile cases of methemoglobinemia, the US Environmental Protection Agency (EPA) set a Maximum Contaminant Level (MCL) for nitrate of 44 mg/L (equal to 10 mg/L nitrogen in nitrate). However, it is now thought that methemoglobinemia *per se* was not caused by nitrate itself, but by fecal bacteria that infected infants and produced NO in their gut. A recent report by Avery has argued that it is unlikely that nitrate causes methemoglobinemia without bacterial contamination, and also that the 40–50 mg/L limit on nitrate in drinking water is not necessary [43]. However, there are now legal limits to the concentrations of nitrate and nitrite in both food and drinking water. The WHO showed that the Acceptable Daily Intake for humans (ADI) for nitrate and nitrite were 3.7 and 0.07 mg/kg body weight/day, respectively, which were based on the calculations from the doses of <500 mg of sodium nitrate/kg body weight that were harmless to rats and dogs. The international estimates of nitrate intake from food are 31–185 mg/day in Europe and 40–100 mg/day in the United States [44,45]. However, the Ministry of Health, Labour and Welfare of Japan reported that the average intake of nitrate in the Japanese populations is around 200–300 mg/day, which is one and a half times to two times the ADI. Furthermore, according to a report by Hord [28], in which the daily nitrate and nitrite intakes were calculated based on the variations using the vegetable and fruit components of the DASH (Dietary Approaches to Stop Hypertension) dietary pattern [46], the level easily exceeds 1,200 mg/day nitrate. This is more than five-fold higher than the WHO's ADI of 3.7 mg nitrate/kg body weight/day, and more than two-fold the US Environmental Protection Agency's level of 7.0 mg nitrate/kg body weight/day for a 60 kg individual [28]. Furthermore, as indicated in Figure 2, approximately 25% of the ingested nitrate is secreted in saliva, and 20% of the secreted nitrate in saliva is converted to nitrite by commensal bacteria on the tongue [22], indicating that about 5% of the originally ingested nitrate is swallowed into the stomach (Figure 1). Therefore, for a DASH diet containing 1200 mg nitrate, an individual would be expected to swallow approximately 45 mg of nitrite a day, which easily exceeds

the ADI of nitrite. Therefore, a comprehensive reevaluation of the health effects of dietary sources of nitrate/nitrite might be required in the near future [28]. Another major health concern regarding dietary nitrate/nitrite is whether dietary nitrate can cause cancer. In fact, nitrate and nitrite themselves are not carcinogenic, but nitrite which is formed from dietary nitrate might react with dietary amines to form carcinogenic nitrosoamines. This phenomenon will be discussed in detail below.

4. Protective Effects of Dietary Nitrate/Nitrite on Lifestyle-Related Diseases

Lifestyle-related disease is a chronic disease characterized by oxidative and proinflammatory state with reduced NO bioavailability [47]. The cellular redox balance in these patients shifts toward a more oxidizing state which affects a number of protein functions at the transcriptional and posttranslational levels, consequently disrupting the cellular homeostasis [11,40]. However, increased NO bioavailability can improve the intracellular redox environment by *S*-nitrosylation-mediated modulation of most classes of proteins present in all cells [9,40]. Recently, accumulating evidence has suggested that dietary nitrate/nitrite improves the features of lifestyle-related diseases by enhancing NO availability, and thus provides potential options for prevention and therapy for these patients [28]. Based on the recent evidence, the beneficial effects of a diet rich in these components are discussed below, focusing on insulin resistance, hypertension, cardiac ischemia/reperfusion injury, chronic obstructive pulmonary disease (COPD), cancer, and osteoporosis.

4.1. Insulin Resistance

The insulin receptor shares a signaling pathway with the activation of endothelial NOS (eNOS) [19,48–52] to regulate the postprandial blood flow and efficient nutrient disposition to peripheral tissues (Figure 4). Therefore, insulin resistance is always associated with impaired NO availability, suggesting that a reciprocal relationship exists between insulin activation and endothelial function [50,53]. Insulin resistance is improved by NO at various levels including insulin secretion [54,55], mitochondrial function [56], modulation of inflammation [57], insulin signaling [58] and glucose uptake [59]. For example, insulin-stimulated NO production has physiological consequences resulting in capillary recruitment and increased blood flow in skeletal muscle, leading to efficient glucose disposal [52].

However, the most important mechanism to improve insulin resistance might be at the post-receptor level of insulin signaling [60] (Figure 4). In diabetic states, increased adiposity releases free fatty acids and produces excessive reactive oxygen species (ROS) through a toll-like receptor 4 (TLR4)-mediated mechanism, which activates a number of kinases and phosphatases [61], and then disrupts the balance of protein phosphorylation/dephosphorylation associated with insulin signaling [62]. The mechanisms underlying the NO-mediated beneficial effects on insulin resistance are as follows (Figure 4): First, NO suppresses the TLR4-mediated inflammation and ROS production by inactivating IkB kinase-β/nuclear factor-κB (IκκB/NF-κβ) [9,63], the main trigger for the induction of a number of proinflammatory cytokines. Second, Wang *et al.*, indicated that NO mediates the *S*-nitrosylation of protein-tyrosine phosphatase 1B (PTPB1) and enhances the effects of insulin [52]. Because PTPB1 dephosphorylates the insulin receptor and its substrates, attenuating the insulin effect, its phosphatase activity tends to be suppressed by eNOS-mediated *S*-nitrosylation. In contrast, when the vascular eNOS activity is impaired, PTPB1 suppresses the downstream signaling to PI3K/Akt, leading to insulin resistance. Therefore, NO might act as a key regulatory mediator for the downstream signaling linking glucose transporter 4 (GLUT4) translocation and glucose uptake [58,64]. Third, Jiang recently reported that NO-dependent nitrosylation of GLUT4 facilitates GLUT4 translocation to the membrane for glucose uptake, and improves insulin resistance [65]. Fourth, excess nutrients also overproduce superoxide in the mitochondrial respiratory chain, leading to the subsequent formation of ROS. NO can inhibit mitochondrial ROS production through the *S*-nitrosylation of mitochondrial respiratory chain complex 1 enzyme and by improving the efficiency of oxidative phosphorylation in the mitochondria [12].

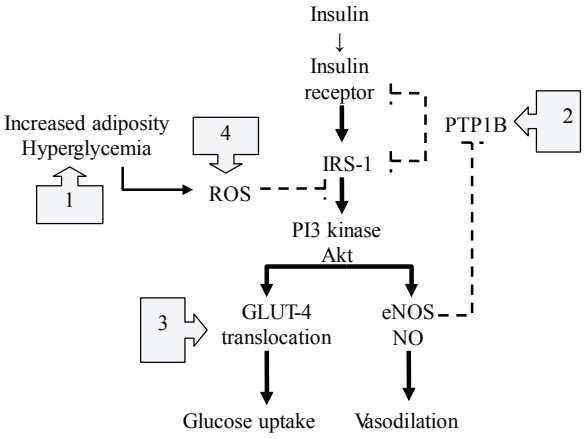

Figure 4. The NO-mediated actions on insulin signaling pathway. The boxes with arrows indicate the sites of NO-mediated actions against insulin resistance. Dotted lines represent inhibition, and solid lines represent stimulation. (**1**) NO suppresses TLR4-mediated inflammation and ROS production; (**2**) NO enhances the effects of insulin through the *S*-nitrosylation-mediated inhibition of phosphatase activity of PTPB1; (**3**) NO-dependent nitrosylation of GLUT4 facilitates glucose uptake; (**4**) NO inhibits mitochondrial ROS production through *S*-nitrosylation of the mitochondrial respiratory chain complex. IRS-1: insulin receptor substrate-1, ROS: reactive oxygen species, NO: nitric oxide, eNOS: endothelial NO synthase, GLUT4: glucose transporter 4, PI3 kinase: phosphatidylinositol 3-kinase, PTPB1: protein-tyrosine phosphatase B1, TLR4: toll like receptor 4.

Indeed, the therapeutic potential of dietary nitrate/nitrite has been supported by recent studies demonstrating the improvements of insulin resistance in humans and animals as a result of its enhancing the NO availability in plasma and tissues [65–68]. As mentioned above, insulin resistance always accompanies metabolic and endothelial dysfunction, which lead to hypertension and atherosclerosis [50,51,53,69,70]. Enhancement of the availability of NO might therefore be a promising strategy for the prevention and treatment of patients with not only insulin resistance, but also endothelial dysfunction [71].

4.2. Hypertension

Increased consumption of fruits and vegetables is associated with a reduction of the risk of cardiovascular disease [72–74]. The DASH studies recommended the consumption of diets rich in vegetables and low-fat dairy products to lower blood pressure, and these effects are thought to be attributable to the high calcium, potassium, polyphenols and fiber and low sodium content in these food items [75,76]. However, vegetable diets containing high nitrate levels increase the plasma levels of nitrate and nitrite [77], which are the physiological substrates for NO production. Accumulating evidence has recently indicated that the nitrate/nitrite content of the fruits and vegetables could contribute to their cardiovascular health benefits in animals [29,33,78–83] and humans [31,84–86].

A number of publications have demonstrated that dietary nitrate reduces blood pressure in humans [87–89]. Larsen *et al.*, reported that the diastolic blood pressure in healthy volunteers was reduced by dietary sodium nitrate (at a dose of 0.1 mmol/kg body weight per day) corresponding to the amount normally found in 150 to 250 g of a nitrate-rich vegetable, such as spinach, beetroot, or lettuce [84]. Webb *et al.*, studied the blood pressure and flow-mediated dilation of healthy volunteers, and showed that the vasoprotective effects of dietary nitrate (a single dose of 500 mL of beetroot juice containing 45.0 $\pm$ 2.6 mmol/L nitrate), were attributable to the activity of nitrite converted from the ingested nitrate [86]. Kapil *et al.*, also showed a similar finding that consuming 250 mL of beetroot juice

(5.5 mmol nitrate) enhanced the plasma levels of nitrite and cGMP with a consequent decrease in blood pressure in healthy volunteers, indicating that there was soluble guanylate cyclase-cGMP-mediated vasodilation following a conversion of the nitrite to bioactive NO [85]. They later presented the effects of dietary nitrate on hypertension, and showed the first evidence that daily dietary nitrate supplementation (250 mL of beetroot juice daily) for four weeks reduced the blood pressure, with improvements in the endothelial function and arterial stiffness in patients with hypertension [90]. Because arterial vascular remodeling is the major histological finding associated with aging, these vascular structural changes represent vascular wall fibrosis with increased collagen deposits and reduced elastin fibers, which result in arterial stiffening and subsequent hypertension in elderly patients. Sindler *et al.*, recently demonstrated that dietary nitrite (50 mg/L in drinking water) was effective in the treatment of vascular aging in mice, which was evidenced by a reduction of aortic pulse wave velocity and normalization of NO-mediated endothelium-dependent dilation. They showed that these improvements were mediated by reduction of oxidative stress and inflammation, which were linked to mitochondrial biogenesis and health as a result of increased dietary nitrite. These beneficial effects were also evident with dietary nitrate in their study [91], suggesting that dietary nitrate/nitrite may be useful for the prevention and treatment of chronic age-associated hypertension.

In addition, hypertension is also a major cause of ischemic heart and cardiac muscle remodeling, which lead to congestive heart failure. Bhushan *et al.*, reported that dietary nitrite supplementation in drinking water (50 mg/L sodium nitrite, for nine weeks) increased the cardiac nitrite, nitrosothiol, and cGMP levels, which improved the left ventricular function during heart failure in mice with hypertension produced by transverse aortic constriction. They also showed that dietary nitrite improved the cardiac fibrosis associated with pressure-overloaded left ventricular hypertrophy through NO-mediated cytoprotective signaling [92]. Although a number of studies on the acute effects of dietary nitrate have been conducted using animal models and healthy humans, more evidence in patients with hypertension, as well as additional studies on the long-term effects of dietary nitrate, will be needed in the future.

4.3. Cardiac Ischemia/Reperfusion Injury

During heart ischemia, ATP is progressively depleted in cardiac muscle cells, which impairs ion pumps, leads to the accumulation of calcium ion, and consequently damages the cell membrane stability. On reperfusion, the cardiac muscle cells are further injured, because in the mitochondria, ROS are produced in large quantities due to massive electron leaks and the formation of superoxide with the resupplied oxygen, which denatures cytosolic enzymes and destroys cell membranes by lipid peroxidation. ROS-mediated dysfunction of the sarcoplasmic reticulum also induces massive intracellular calcium overload, leading to the opening of the mitochondrial permeability transition pore and causing cell apoptosis or necrosis, depending on the intracellular ATP levels [93,94]. The availability of vascular NO would thus be expected to be impaired due to the reduced NOS activity in ischemia and subsequent consumption by superoxide during reperfusion [95], resulting in severe ischemia/reperfusion injury [30].

Nitrite, nitrate, and NO-related compounds (e.g., *S*-nitrosothiols) are constitutively present in blood and tissues. The nitrite level in cardiac tissue is a couple of times higher than that in plasma due to an unknown form of active transport from blood to tissues or due to the oxidation of endogenously generated-NO to nitrite by ceruloplasmin [96], and serves as a significant extravascular pool for NO during tissue hypoxia [97]. Carlström *et al.*, showed that dietary nitrate increased the tissue levels of nitrite and *S*-nitrosothiols in the heart, and attenuated oxidative stress and prevented cardiac injury in Sprague-Dawley rats subjected to unilateral nephrectomy and a high-salt diet [37]. Shiva *et al.*, recently showed that the nitrite stored in the heart and liver via systemic and oral routes augmented the tolerance to ischemia/reperfusion injury in the mouse heart and liver [33].

Although the genetic overexpression of eNOS in mice attenuates myocardial infarction [98], in general, the protective effects of NO on cardiac ischemia/reperfusion injury depend on the local stock

of nitrite and its subsequent reduction to NO at the critical moment when NOS activity is lacking under hypoxic conditions. Indeed, the tissue levels of *S*-nitrosothiols (NO-mediated signaling molecules) are enhanced through the nitrite reduction due to NOS inhibition, hypoxia, and acidosis [97], suggesting that the tissue nitrite stores can be regarded as a backup and on-demand NO donor. There are a number of factors that have been demonstrated to reduce nitrite in the tissues, including deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, heme-based enzymes in the mitochondria and acidosis during ischemia [99,100]. In patients with coronary heart disease, the different consequences of myocardial infarction may depend on the patient's daily intake of nitrate/nitrite. Indeed, Bryan *et al.*, showed that dietary nitrite (50 mg/L) or nitrate (1 g/L) supplementation in drinking water for seven days maintained higher steady-state levels of nitrite and nitroso compounds, as well as nitrosyl-heme, in mouse cardiac muscle, and these mice exhibited a smaller cardiac infarct size after ischemia/reperfusion injury compared with control mice fed a diet deficient in nitrate/nitrite for seven days. These findings suggest that this protective nitrate/nitrite may be derived at least in part from dietary sources [29].

Shiva *et al.*, demonstrated that the cytoprotective effects of nitrite on ischemia/reperfusion injury are mediated by post-translational *S*-nitrosylation of complex 1 in the mitochondrial respiratory chain, which consequently inhibits the overall mitochondrial ROS formation and apoptotic events [101]. Another possible cytoprotective effects of nitrite may be mediated by the effects of *S*-nitrosylation on the intracellular Ca^{2+} handling, which decreases Ca^{2+} entry by inhibiting L-type Ca^{2+} channels and increasing the sarcoendoplasmic reticulum (SR) Ca^{2+} uptake by activating SR Ca^{2+} transport ATPase (SERCA2a) [102]. These effects will lead to an attenuation of the increase in cytosolic Ca^{2+} during ischemia and Ca^{2+} overload during reperfusion.

Intriguingly, recent large-scale epidemiological studies reported the preventive effects of antioxidant supplementations including vitamins E, C, and beta carotene rich in fruits and vegetables on cardiovascular disease, whereas no beneficial effects were shown in other studies, and in some cases a decrease in cardiovascular protection with these supplementations was observed [103–105]. On the other hand, a number of epidemiological studies have shown the preventive effects of fruits and vegetables on coronary heart disease [3–6,106]. It should be noted that the consumption of an appropriate amount of fruits and vegetables, which might contain balanced doses of nitrate/nitrite and vitamins, might be more effective with regard to health maintenance and improvement than antioxidant supplementation alone.

4.4. Chronic Obstructive Pulmonary Disease (COPD)

COPD is considered to be a lifestyle-related disease, because long-term tobacco smoking and subsequent chronic bronchitis are causally associated with this disease [107]. Varraso *et al.*, recently reported the importance of a healthy diet in multi-interventional programs to prevent COPD [108]. They showed that high intake of whole grains, polyunsaturated fatty acids, nuts, and long chain omega-3 fats, and low intake of red/processed meats, refined grains and sugar-sweetened drinks, were associated with a lower risk of COPD in both women and men.

Because cured meats such as bacon, sausage and ham contain high doses of nitrite for preservation, antimicrobial and color fixation, epidemiological studies have demonstrated that the consumption of cured meats is positively linked to the risk of newly diagnosed COPD [109–111]. Nitrite generates reactive nitrogen species, which may cause nitrosative damage to the lungs, eventually leading to structural changes like emphysema [111]. This is supported by an animal study in which rats chronically exposed to 2000 and 3000 mg/L of sodium nitrite in their drinking water for two years showed distinct lung emphysema [112]. However, the dose of nitrite used in that study was 250–350 mg/kg/day, which was too high to compare with those achieved in standard human diets [113].

In fact, cured meats have been reported to generally comprise only 4.8% of the daily nitrite intake, and surprisingly, 93% of the total ingestion of nitrite is derived from saliva [114], suggesting that cured meats provide minimal contributions to the human intake of nitrite, even if they are frequently

consumed. In addition, the recent nitrite levels in processed meats have been approximately 80% lower than those in the mid-1970s in the US [115]. Therefore, discussions encompassing all ingested sources of nitrite should consider whether or not the nitrite derived only from the consumption of cured meats might be responsible for the development of COPD.

On the other hand, a number of epidemiological studies have shown the beneficial effects of *n*-3 fatty acids, vitamins, fruits and vegetables on lung functions and the risk of COPD [108,116–122]. Although it may be difficult to isolate the specific effects of these dietary nutrients, as discussed above, the nitrate and nitrite derived from vegetables and fruits are reduced to NO, which is followed by the formation of *S*-nitrosothiols [123], rather than the formation of nitrosamines especially in the presence of reducing agents such as vitamin C and E in the stomach [28,124]. It has been shown that high dietary nitrate intake does not cause the expected elevation of the gastric nitrite concentrations or appreciable changes in the serum nitrite concentrations [125].

As mentioned above, different from the effects of the direct elevation of nitrite concentration in the plasma, the entero-salivary route of dietary nitrate/nitrite might enhance the availability of NO through the formation of *S*-nitrosothiols and its transnitrosylation to the other thiol residues of proteins, suggesting that, depending on the tissues and organs, separate metabolic pathways might exist for NO availability in this entero-salivary route. Consistent with this idea, Larsen *et al.*, recently demonstrated that acute intravenous infusion of nitrite enhanced the plasma levels of nitrite, whereas it did not affect the oxygen consumption (VO_2) or the resting metabolic rate (RMR) in humans. Instead, dietary nitrate significantly reduced the VO_2 and RMR by improving the mitochondrial respiratory chain function and enhancing efficient O_2 consumption, suggesting that rather than direct nitrite infusion to enhance the plasma nitrite levels, biologically active nitrogen oxide (including the *S*-nitrosothiols produced in the stomach) might be an important molecule for the transfer of biological NO activity for cardiopulmonary function [126]. Because COPD is a state of protein-energy malnutrition due to an increased resting metabolic rate and VO_2, the effects of dietary nitrate on the reduction of the RMR and VO_2 might be advantageous for patients with COPD.

Whether the role of NO in COPD is protective or pathogenic depends on the origin and concentration range of NO. NO activity derived from dietary nitrate and constitutive NOS might be protective against COPD largely through the *S*-nitrosothiol-mediated mechanism including inhibition of the noncholinergic nonadrenergic nerve activity, bronchial smooth muscle relaxation, reduction of airway hyperresponsiveness, downregulation of the proinflammatory activity of T lymphocytes, and antimicrobial defense [127]. However, the deleterious effects of NO on the development of COPD might be derived from iNOS-mediated pro-inflammatory signaling [128], which is consequently (not causally) reflected by the huge amount of NO in the exhaled air of patients with COPD [129].

Recent human studies have demonstrated that dietary nitrate (beetroot juice containing approximately 200–400 mg of nitrate) improved the exercise performance and reduced blood pressure in COPD patients [130,131]. However, large-scale epidemiological evidence of the impact of nitrate is still lacking.

4.5. Cancer

In the stomach, swallowed nitrite is decomposed to form a variety of nitrogen compounds, including *N*-nitrosoamines [132]. In the 1950s, Magree *et al.*, first reported that *N*-nitrosodimethylamine caused malignant primary hepatic tumors in rats [133]. After this report, a number of studies followed in relation to the carcinogenic effects of *N*-nitroso compounds in animal models [134,135]. In particular, the dietary intake of red and cured meats was found to be associated with an increased risk of certain types of cancer due to the relatively large amounts of nitrite added. However, the methodological aspects have been challenged concerning the high dose of nitrosatable amines, and the physiological difference between animals and humans [136].

In the stomach, the nitrosonium ion (NO^+) derived from nitrite can bind to thiol compounds (R-SH) and amines (especially secondary amines: R_1-NH-R_2), forming *S*-nitrosothiol and

N-nitrosamine, respectively. However, while *N*-nitrosamine formation occurs even at neutral or basic pH, *S*-nitrosothiol formation tends to occur only under acidic conditions. In addition, this reaction kinetically occurs much more easily than *N*-nitrosamine formation, particularly in the presence of vitamins C and E and polyphenols, which are highly present in fruits and vegetables, which also eliminate potent nitrosating agents such as the N_2O_3 formed from nitrite by decomposing them to NO. This might partly explain why patients with achlorhydria and non-vegetarians eating large amounts of cured meats are at risk of developing gastric cancer [137–142].

However, this idea appears to be inconsistent with the belief that dietary nitrite is a major cause of cancer. This is because, according to the average nitrate/nitrite intake of adults in the US, most of the daily nitrate intake (around 90%) comes from vegetables, and the nitrite intake is primarily derived from recycled nitrate in the saliva (5.2–8.6 mg/day nitrite), with very little coming from cured meats (0.05–0.6 mg/day nitrite in 50g/day cured meats) and other dietary sources (0–0.7 mg/day nitrite) [136], suggesting that the entero-salivary route may be the more important source of nitrosamine exposure than exogenous intake including cured meats, that is, spitting out saliva all day long might prevent cancer development more effectively than cutting cured meats. However, recent experimental and epidemiological studies could not demonstrate a positive relationship between nitrate consumption and the risk of cancer [121,134], and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives concluded in 2008 that there was no evidence that nitrate was carcinogenic in humans. Consistent with this, recent studies have found no link between dietary nitrate and cancer [143,144].

Bradbury *et al.*, reported a large-scale study (>500,000 participants) of the associations between fruit, vegetable, or fiber consumption and the risk of cancer at 14 different sites. They showed that there was an inverse association between fruit intake and the risk of upper gastrointestinal tract and lung cancer, as well as an inverse association between fiber intake and liver cancer. The dietary intake of vegetables, as well as fruits and fiber, was inversely associated with the risk of colorectal cancer, suggesting that there is little evidence that vegetable intake is associated with the risk of any of the individual cancer sites reviewed [145].

However, chronic inflammation, including inflammatory bowel disease and *Helicobacter pylori*-induced gastritis induce inducible NOS (iNOS) and generate large quantities of NO [22,146,147], forming nitrosating and oxidant species such as N_2O_3 and peroxynitrite, which might cause mutagenesis through deamination, nitration of DNA, or inhibition of the DNA repair system [148,149]. Depending on the sites and amounts of NO generation, NO might represent a double-edged sword in the sense that it confers both protective and deleterious effects on cancer development [150,151].

Meta-analyses of primary and secondary cancer prevention trials of dietary antioxidant supplements, such as beta carotene, vitamins A, C, and E, showed a lack of efficacy, and on the contrary, an increased risk of mortality [104]. Although the general role of NO in carcinogenesis is complicated, and many unknown mechanisms remain to be resolved, the dietary nitrate/nitrite (at least that obtained from plant-based foods such as fruits and vegetables) have obvious inhibitory effects on cancer risk by playing some synergistic role with other nutrients in these foods.

4.6. Osteoporosis

Lifestyle habits, such as smoking, alcohol intake, little or no exercise, and an inadequate amount of calcium intake all influence the calcium-vitamin D metabolism [152–155] and bone mineral density, in some cases leading to osteoporosis, particularly in postmenopausal women [156]. The implications of NOS-mediated NO in the regulation of bone cell function have been well described in a number of publications [157]. For example, iNOS-induced NO production following stimulation with proinflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α), inhibits bone resorption and formation, resulting in osteoporosis in patients with inflammatory diseases such as rheumatoid arthritis [158]. On the other hand, eNOS, a constitutive NO synthase, plays an important role in regulating osteoblast activity and bone formation, because eNOS knockout mice

exhibit osteoporosis due to defective bone formation, and eNOS gene polymorphisms were reported to be causally linked to osteoporosis in postmenopausal women [159].

In addition, Wimalawansa *et al.*, showed that some of the beneficial effects of estrogen on bone metabolism are mediated through a NO-cGMP-mediated pathway [160], suggesting that NO donor therapy might provide a promising alternative to estrogen therapy. In this context, it has been shown that organic nitrate NO donors, such as glycerol trinitrate, isosorbide dinitrate and mononitrate all have beneficial effects on experimental and clinical osteoporosis [161–163], and a number of epidemiological studies also indicated that a high fruit and vegetable intake appears to have a protective effect against osteoporosis in men and pre- and postmenopausal women [164–166]. However, few studies have been conducted to evaluate the detailed mechanism by which inorganic nitrate/nitrite prevents osteoporosis at the molecular level, and thus further basic research will be needed for this purpose.

5. Conclusions

Dietary nitrate, which is provided by fruits and vegetables, can transmit NO activities in various molecular forms, including NO, nitrite, and *S*-nitrosothiols, through the entero-salivary pathway. Although the role of diet-derived NO activity in lifestyle-related diseases is complex and remains to be fully elucidated, the intake of nitrate as a nutrient in vegetables might be beneficial to human health as a result of synergistic effects with other nutrients present in vegetables, and would be recommended as a nutritional approach to the prevention and treatment of the lifestyle related diseases.

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Section 2:
Diet and Glucose Homeostasis

nutrients

MDPI

Article

Efficacy of Aloe Vera Supplementation on Prediabetes and Early Non-Treated Diabetic Patients: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Abstract: The aim of this study was to evaluate evidence for the efficacy of aloe vera on managing prediabetes and early non-treated diabetes mellitus. We performed a systematic search of PubMed, Embase, and Cochrane Central Register of Controlled Trials until 28 January 2016. A total of five randomized controlled trials (RCTs) involving 415 participants were included. Compared with the controls, aloe vera supplementation significantly reduced the concentrations of fasting blood glucose (FBG) ($p = 0.02$; weighed mean difference [WMD]: −30.05 mg/dL; 95% confidence interval [CI]: −54.87 to −5.23 mg/dL), glycosylated hemoglobin A1c (HbA1c) ($p < 0.00001$; WMD: −0.41%; 95% CI: −0.55% to −0.27%), triglyceride ($p = 0.0001$), total cholesterol (TC) ($p < 0.00001$), and low density lipoprotein-cholesterol (LDL-C) ($p < 0.00001$). Aloe vera was superior to placebo in increasing serum high density lipoprotein-cholesterol (HDL-C) levels ($p = 0.04$). Only one adverse event was reported. The evidence from RCTs showed that aloe vera might effectively reduce the levels of FBG, HbA1c, triglyceride, TC and LDL-C, and increase the levels of HDL-C on prediabetes and early non-treated diabetic patients. Limited evidence exists about the safety of aloe vera. Given the small number and poor quality of RCTs included in the meta-analysis, these results are inconclusive. A large-scale, well-designed RCT is needed to further address this issue.

Keywords: aloe vera; prediabetes; randomized controlled trials; meta-analysis

1. Introduction

Diabetes mellitus is a group of metabolic disorders that is one of the major global health threats to humans due to its increasing prevalence, disabling complications, and chronic course [1]. Both impaired glucose tolerance and impaired fasting glucose are called "prediabetes", which does not reach the diagnostic cutoff values that would precipitate a diagnosis of type 2 diabetes mellitus (T2DM) [2]. It is predicated that approximately 5%–10% of the prediabetic population would suffer from diabetes after approximately a year, and the number of prediabetes patients could reach up to 470 million globally by 2030 [3]. Thus, preventing or delaying the onset of clinical T2DM in prediabetic subjects is a reasonable way to combat the diabetes epidemic and to lessen healthcare costs. Current medications to control blood glucose may have dangerous side effects over time, such as increased risk of liver toxicity, weight gain, and cardiovascular diseases [4]. Therefore, it is not surprising that complementary medicines and natural products, such as oats, are gaining increasing popularity among patients with hyperglycemia, due to affordability and fewer side effects [5]. Many traditional remedies

for diabetes mellitus use plant sources, and over 400 species were reported to display hypoglycemic effects, although few of them have been evaluated [6,7].

Aloe is a succulent plant belonging to the Liliaceal family, of which there are more than 200 species found worldwide [8]. Aloe vera is a common name for *Aloe barbadensis*, which is the most widely-used species of aloe. Aloe vera has had applications in health and cosmetic products for many centuries, as well as anti-tumor, antioxidant, anti-inflammatory, and laxative properties [9,10]. It includes over 75 active ingredients that contain enzymes, vitamins, sugars, minerals, lignin, amino acids, and salicylic acid, and most of the constituents appear to be of biological importance in curing diseases [11].

The hypoglycemic effects of aloe vera have been investigated by various researchers. However, there is no consensus on the beneficial effects of aloe vera supplementation in the preventing or improvment of metabolic-syndrome-related disorders [12]. Some studies with rodents have demonstrated the hypoglycemic effect of aloe vera, whereas other studies have shown no significant effects [13,14]. Similarly, there is growing evidence that aloe vera derived extracts showed a preventive effect against insulin resistance and a lipid-lowering effect; however, there is still a great deal of controversy about these data, which have made it difficult to draw any definitive conclusions [14,15]. Therefore, we conducted a meta-analysis to quantitatively summarize and critically evaluate the evidence from randomized clinical trials (RCTs) involving the use of aloe vera as a hypoglycemic supplement.

2. Materials and Methods

2.1. Search Strategy

We searched the following three electronic databases from their inception until 28 January 2016 for the identification of studies: PubMed, Embase and Cochrane Central Register of Controlled Trials. Keywords for databases searching were: ("prediabetes" OR "pre-diabetes" OR "prediabetic state" OR "hyperglycemia" OR "borderline diabetes" OR "impaired glucose tolerance" OR "impaired fasting glucose" OR "diabetes prevention" OR "prevention of diabetes" OR "diabetes mellitus" OR "diabetes mellitus type 2" OR "non-insulin dependent diabetes mellitus") AND ("Aloe" OR "Aloe vera" OR "Aloe barbadensis" OR "Aloe barbadensis extract" OR "Aloe vera extract"). All of the indexed studies were retrieved, and the reference lists of the identified publications were reviewed for additional pertinent studies. No language restriction was applied for searching. The literature search and study selection were carried out independently by two reviewers (Y.Z. and W.L.) with a standardized approach. Any inconsistencies were resolved by consultation with a third reviewer (D.L.).

2.2. Inclusion Criteria

The diagnostic of prediabetes or T2DM was based on the definitions described by the World Health Organization [16] or the American Diabetes Association [4]. Patients were not limited by age, sex, race, or body size. The inclusion criteria were as follows: (1) randomized controlled trials; (2) studies were included irrespective of whether or not they incorporated lifestyle changes into their trial regimen; (3) studies involving the use of aloe vera as part of a combination product or treatment package were excluded from the systematic review; (4) no history of any hypoglycemic medication use; (5) no history of other diseases like coronary heart disease, stroke, cancer, hepatic disorder, a chronic inflammatory condition, or psychiatric disorders; and (6) primary outcomes reported included at least glucose and/or lipid profile.

2.3. Data Extraction and Quality Assessment

One reviewer screened the titles and abstracts of the RCTs that we identified. Full text articles were obtained for those trials that fulfilled the inclusion criteria or for which sufficient information was given. Two reviewers (Y.Z. and W.L.) independently extracted data from trials that met the inclusion criteria on an Excel spreadsheet; any discrepancies in extracted data were resolved by a group discussion and consensus and final arbitration by the Cochrane editorial base. Attempts were

made to seek further information from the authors of the original studies if data were unclear or incomplete. The methodologic quality criteria of randomization, allocation concealed, blinding, and intention-to-treat (ITT) analyses were graded as adequate, inadequate, and unclear. Studies were categorized as double blinding, single blinding, or unclear.

2.4. Statistical Analysis

In our meta-analysis, glucose metabolism, such as fasting blood glucose (FBG), insulin, and glycosylated hemoglobin A1c (HbA1c), were considered primary outcomes. Because diabetes was strongly associated with an increased risk of cardiovascular disease, and often coexists with dyslipidemia, other secondary outcomes included lipid profile, such as triglyceride, total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), and high density lipoprotein-cholesterol (HDL-C). All outcomes extracted from the literature were continuous data.

Review Manager soft-ware 5.2 provided by the Cochrane Collaboration was used for the meta-analysis. For continuous data, weighted mean differences (WMDs) or standardized mean differences (SMDs) with their 95% confidence intervals (95% CIs) were calculated. Analyses were separately performed for each outcome. Heterogeneity across studies was assessed by the Cochrane's Q-test. The fixed-effects model was used to calculate the total effect size where Cochrane's Q-test $p > 0.10$ and the I^2 statistic $I^2 < 50\%$ indicated statistical homogeneity. If heterogeneity of $p < 0.10$ or $I^2 > 50\%$ was found among the trials, a random-effects model was chosen. Two-tailed p-values $\leqslant 0.05$ or 95% CIs not containing 0 (WMD) were considered statistically significant. Publication bias was not assessed because the number of the included trials was less than 10.

3. Results

3.1. Eligible Studies and Baseline Characteristics

We identified 282 records on aloe vera after searching the three databases mentioned above. Finally, a total of five randomized controlled trials [17–21] from 1996 to 2016 containing 415 participants were included (Figure 1). The key characteristics of these studies are outlined in Table 1.

Most of the included RCTs had flaws in the reporting of their methodology. Although randomization was declared in all studies, only two trials reported how the randomization was conducted [19,21], and none of the studies reported adequate allocation concealment. Three of five studies turned out to be double-blinded [18,19,21], and information relating to withdrawal/dropout was reported adequately in three trials [17,19,21]. ITT analysis was conducted in only one study [19].

All included RCTs involved overweight and/or obese participants, except for one study that did not report [17]. Only one adverse event was reported [19]. Participants in two RCTs [18,21] were allowed to continue their normal lifestyle, whereas the remaining studies did not report this information. The duration of follow-up ranged from 6 to 12 weeks. According to comparators used in these studies, our meta-analysis was divided into two parts.

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

Study	Main Diagnoses of Study Participants	Mean FBG (mg/dL)	Mean HbA1c (%)	Mean BMI (kg/m^2)	Gender (Male/ Female)	Randomized/ Analyzed	Daily Dosage (Formulation)	Treatment Duration (Weeks)	Main Outcomes	Adverse Events	Control for Lifestyle Factors	Randomization Appropriate	Allocation Concealed	Sample Size Determined	Groups Similar at Baseline	Blinding	ITT Analysis
Yongchaiyudha, 1996 [17]	Early untreated diabetes	250.7	Unclear	Unclear	50/22	72/70	Two tablespoonful (juice)	6	Glucose and lipid profile	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Single blinding	Unclear
Devaraj, 2013 [18]	Prediabetes	109	5.9	34.7	15/29	45/44	1.0 g (capsules)	8	Glucose and lipid profile	Unclear	Normal diet and exercise at least 100 min per week	Unclear	Unclear	Unclear	Yes	Double blinding	Unclear
Choi, 2013 [19]	Prediabetes and early untreated diabetes	116.2	6.2	27.4	96/40	136/122	2.8 g (capsules)	8	Glucose, lipid profile and obesity-related biomarkers	One adverse event	Unclear	With the randomization code generated by software	Unclear	Unclear	Yes	Double blinding	Inadequate
Choudhary, 2014 [20]	Untreated diabetes	130.8	Unclear	Unclear	90/0	90/90	0.2 g (powder)	12	Glucose, lipid profile and blood pressure	Unclear	Unclear	Unclear	Unclear	Unclear	Yes	Unclear	Unclear
Alinejad-Mofrad, 2015 [21]	Prediabetes	111.1	6	28.2	21/49	72/70	1.0 g (capsules)	8	Glucose and lipid profile	Unclear	Normal diet and exercise	Blocking randomization	Unclear	Unclear	Yes	Double blinding	Unclear

BMI: body mass index; FBG: fasting blood glucose; HbA1c: hemoglobin A1c; ITT: intention to treat.

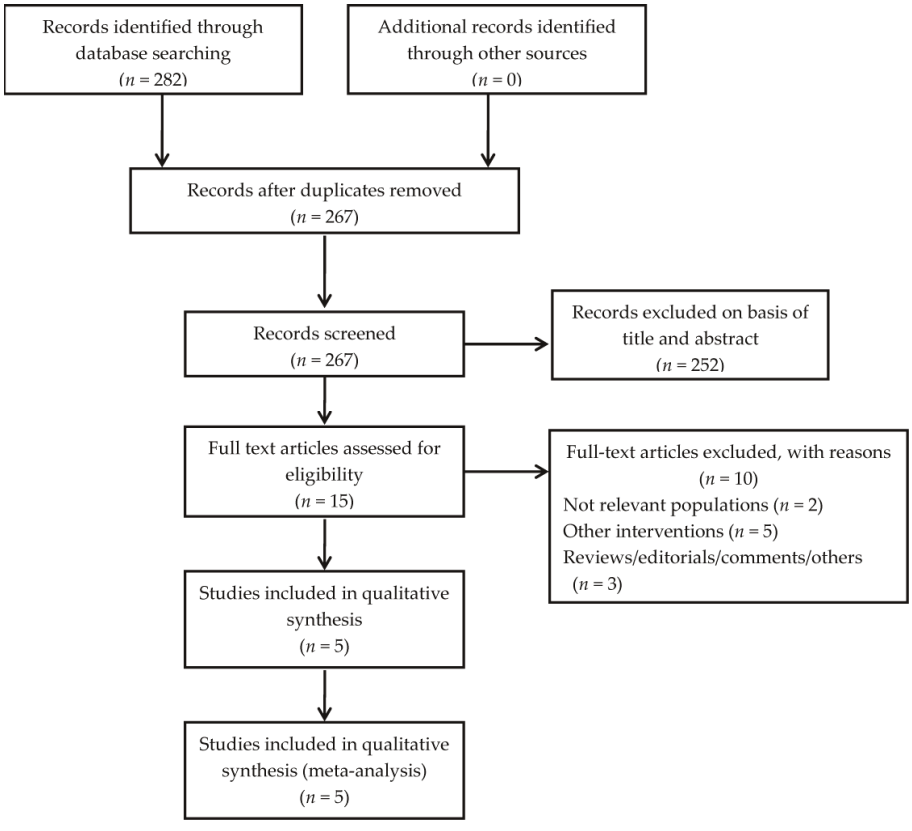


Figure 1. Flow diagram for study identification.

3.2. Meta-Analyses of Primary Outcomes

The changes in FBG were evaluated in the five studies [17–21], which included 328 cases. However, significant heterogeneity was observed among these studies ($p < 0.00001$, $I^2 = 100\%$). The random effects model of meta-analysis was used to combine the effect size. Aloe vera was superior to placebo in reducing FBG levels ($p = 0.02$; WMD: −30.05 mg/dL; 95% CI: −54.87 to −5.23 mg/dL). Two studies compared the effects of aloe vera and placebo on insulin [18,19]. There were no significant changes in the concentration of insulin ($p = 0.15$; SMD: −1.71; 95% CI: −4.07 to 0.64), with heterogeneity existing among these studies ($p < 0.00001$, $I^2 = 96\%$). The changes in HbA1c were evaluated in the two studies [18,21]. Analysis of aggregated data showed a significant reduction in HbA1c ($p < 0.00001$; WMD: −0.41%; 95% CI: −0.55 to −0.27%), without evidence of heterogeneity ($p = 0.61$, $I^2 = 0\%$; Figure 2).

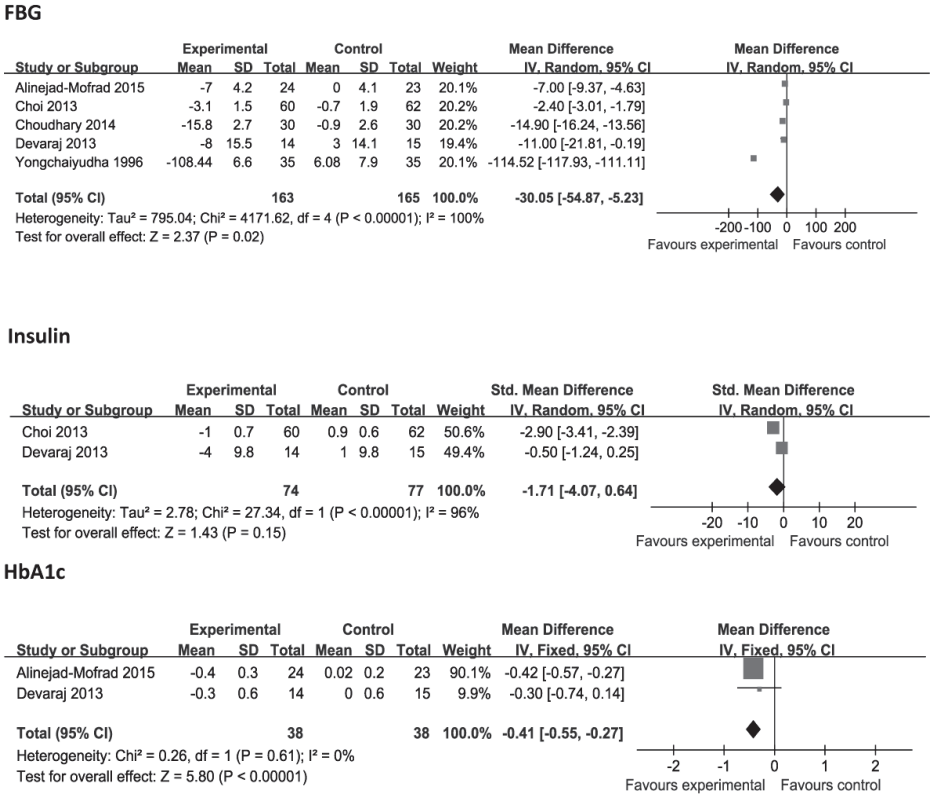
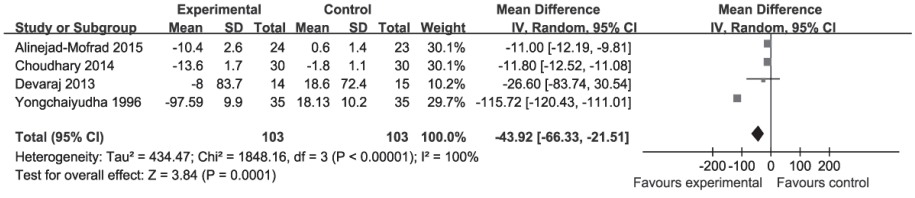


Figure 2. Forest plot of studies that evaluated the effect of aloe vera on primary outcomes compared with placebo. Each block represents a study. Size of square is proportional to the precision of the estimate. Each square represents the weighted mean difference (WMD) or standardized mean difference (SMD) for each study with 95% confidence interval (CI) indicated by horizontal line. FBG, fasting blood glucose; HbA1c, hemoglobin A1c.

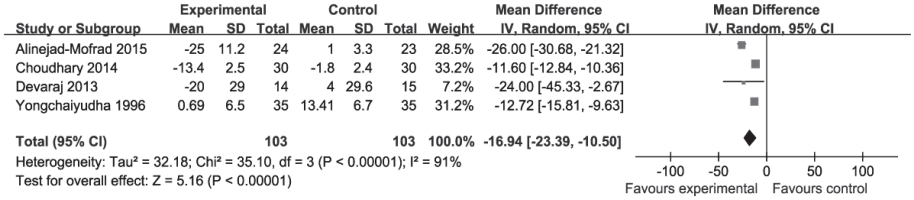
3.3. Meta-Analyses of Secondary Outcomes

Four studies [17,18,20,21], which included 206 cases, compared the effects of aloe vera and placebo on triglyceride and TC levels. After analysis of the aggregated results, we found that aloe vera was superior to placebo in reducing serum triglyceride and TC levels ($p = 0.0001$; WMD: −43.92 mg/dL; 95% CI: −66.33 to −21.51 mg/dL and $p < 0.00001$; WMD: −16.94 mg/dL; 95% CI: −23.39 to −10.50 mg/dL, respectively), although heterogeneity existed among these studies ($p < 0.00001$, $I^2 = 100\%$ and $p < 0.00001$, $I^2 = 91\%$, respectively). The changes in HDL-C and LDL-C levels were evaluated in the three studies [18,20,21]. Aloe vera was superior to placebo in increasing serum HDL-C levels ($p = 0.04$; WMD: 2.67 mg/dL; 95% CI: 0.11 to 5.23 mg/dL), and reducing serum LDL-C levels ($p < 0.00001$; WMD: −13.30 mg/dL; 95% CI: −17.19 to −9.41 mg/dL). High heterogeneity was detected with HDL-C and LDL-C variables ($p = 0.0008$, $I^2 = 86\%$ and $p < 0.00001$, $I^2 = 96\%$, respectively; Figure 3).

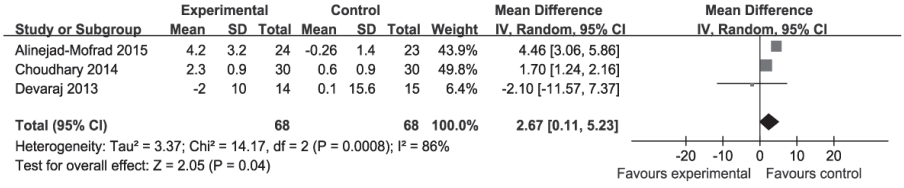
Triglyceride



TC



HDL-C



LDL-C

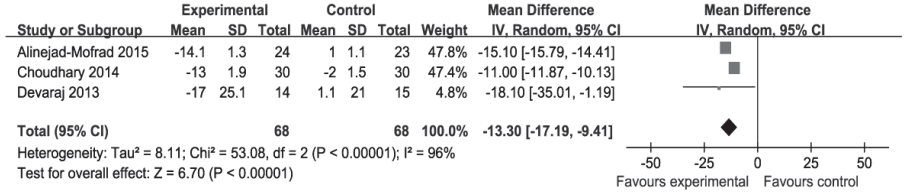


Figure 3. Forest plot of studies that evaluated the effect of aloe vera on secondary outcomes compared with placebo. See Figure 2 for the legend of symbols used. TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

4. Discussion

This study is the first meta-analysis regarding the effects of aloe vera for prediabetes and non-treated diabetic patients that has been conducted to synthesize the results from independent randomized controlled studies to draw an overall conclusion. Although significant differences between groups were found for most parameters, the limited evidence reveal a statistically significant difference in reducing serum FBG and HbA1c levels favoring aloe vera over placebo. In addition, aloe vera has also been shown to reduce the levels of triglyceride, TC and LDL-C, and increase the levels of HDL-C.

Our findings are consistent with some animal studies [13,14]. Several mechanisms may be involved in the association between glucose-lipid metabolism and aloe vera. It could be due to the efficacy of high molecular weight polysaccharides or phytosterols isolated from aloe vera gel

in enhancing glucose transport by modulating the proximal and distal markers involved in glucose uptake and reducing serum concentrations of cholesterol by reducing the absorptions of cholesterol from the gut. Another theory is that the aloe vera extract can lower the level of blood glucose and lipid in diabetic rats by reducing toxic effects of fat in the liver to improve sensitivity of cells to insulin [14,22,23]. It has also been hypothesized that normalization of plasma lipid status by aloe vera may be explained by its ability to suppress adipogenic gene expression, increased clearance and decreased production of the major transporters of endogenously synthesized cholesterol and triglycerides [14]. Additionally, some researchers believe that aloe vera reduce body fat and improve insulin sensitivity by activating adenosine monophosphate-activated muscle protein kinase, which is important in the regulation of glucose and lipid metabolism [24].

Although some reviews have recently been published about the hypoglycemic effects of aloe vera, the quality of these reviews was limited, and they did not present specific methods on data extraction or assessment of heterogeneity. The following factors have strengthened this meta-analysis. First, studies were included or excluded according to strict criteria. Next, the meta-analysis offered an up-to-date and complete overview of all RCTs involving the efficacy of aloe vera supplementation in managing prediabetes and early non-treated diabetic patients, because it was the result of an extensive search, including gray literature and unpublished studies.

There are several limitations should be considered before recommending the findings of this review to clinicians. First, our analysis was based on small number of trials and the backgrounds of patients varied, which would result in low statistical power and the publication bias could not be excluded. Second, insufficient data were available. Insulin or glycosylated HbA1c was not available in the majority of studies, thereby limiting the reliable results. Next, some studies [17,18,20] did not examine or report whether or not blinding requirements were fully met and allocation concealments were fully achieved. ITT analysis was performed in only one study [19]. Hence, selective bias and measurement bias may have existed in the trails. Finally, significant between-study heterogeneity was detected for most of the variables assessed. Three different aloe vera-based preparations were manufactured in five different regions, Thailand, United States, South Korea, India and Iran. Furthermore, these discrepancies in pharmacological effects of various aloe vera preparations may be due to several other factors, including a lack of consistency among studies in relation to standardization of aloe vera manufacturing process, dosage, duration of treatment, units of laboratory tests and races of the selected patients. Such heterogeneity confounds interpretation of statistical findings. We had initially planned to conduct subgroup analyses, however, there were not a sufficient number of trials to perform this analysis. Therefore, the random-effects model was adopted, although it cannot completely eliminate heterogeneity.

Despite these limitations, the present findings could provide useful information on the future research. First, insulin resistance is thought to be a key factor in the development of diabetes; unfortunately, insulin did not appear in a large number of studies outcomes. Second, lifestyle factors, such as food intake and physical exercise, are very important aspects of blood glucose control. However, the difference in the average daily caloric intake and level of physical activity undertaken by study participants was unclear. Most studies lacked objective outcome measures to estimate the extent to which these variations influenced the outcome of the study result. Therefore, focusing on these supplementary clues may be useful in future research studies on the topic. Third, not all of these included aloe vera preparations were assumed to be identical in the composition and biological activity they possessed, which might lead to the discrepancies in the glucose-lowering effect. The variation in daily dosages makes it difficult to determine the minimum effective dose of aloe vera that can cause a blood glucose reduction. Future studies should focus on the effects of aloe preparations, dosage, and the part of the plant used. Finally, the common adverse effects of aloe vera supplementation are abdominal pain, cramping, and muscle weakness [8,9]. However, only one study briefly described the adverse reactions of subjects in the aloe vera group [19]. The selected trials administered aloe vera for 6–12 weeks. Considering the fact that these studies were of short duration, the safety of long-term

aloe vera intake seems uncertain. Thus, it is essential for investigators of future trials to incorporate surveillance time frames into the clinical trials to monitor any medium- and long-term adverse events associated with the use of aloe vera.

5. Conclusions

In conclusion, the currently available data showed that aloe vera might reduce the levels of FBG, HbA1c, triglyceride, TC and LDL-C, and increase the levels of HDL-C in prediabetes and early non-treated diabetic patients; however, limited evidence exists about the safety of aloe vera, given the small RCTs, poor quality of RCTs included, and the considerable heterogeneity seen in the study results, the magnitude of this effect is small and the clinical relevance is uncertain. Large-scale, multi-center and placebo-controlled long-term trials should be rigorously designed to substantiate the current findings and to investigate the long-term effects of aloe vera supplementation on managing prediabetes and T2DM.

Author Contributions: Y.Z. and W.L. contributed equally to this work; Y.Z. and H.T. conceived and designed the study; Y.Z. and W.L. performed data extraction and drafted the paper; Y.Z., W.L., D.L. and T.Z. performed the meta-analysis and discussed study findings; Y.Z., W.L. and H.T. revised the paper for submission for publication.

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

Abbreviations

The following abbreviations are used in this manuscript:

CI	Confidence interval
FBG	Fasting blood glucose
HbA1c	Hemoglobin A1c
HDL-C	High density lipoprotein-cholesterol
ITT	Intention to treat
LDL-C	Low density lipoprotein-cholesterol
RCT	Randomized clinical trial
SMD	Standardized mean difference
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
WMD	Weighed mean difference

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nutrients

MDPI

Review

Dietary Advanced Glycation End Products and Risk Factors for Chronic Disease: A Systematic Review of Randomised Controlled Trials

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Abstract: Dietary advanced glycation end-products (AGEs) form during heating and processing of food products and are widely prevalent in the modern Western diet. Recent systematic reviews indicate that consumption of dietary AGEs may promote inflammation, oxidative stress and insulin resistance. Experimental evidence indicates that dietary AGEs may also induce renal damage, however, this outcome has not been considered in previous systematic reviews. The purpose of this review was to examine the effect of consumption of a high AGE diet on biomarkers of chronic disease, including chronic kidney disease (CKD), in human randomized controlled trials (RCTs). Six databases (SCOPUS, CINHAL, EMBASE, Medline, Biological abstracts and Web of Science) were searched for randomised controlled dietary trials that compared high AGE intake to low AGE intake in adults with and without obesity, diabetes or CKD. Twelve dietary AGE interventions were identified with a total of 293 participants. A high AGE diet increased circulating tumour necrosis factor-alpha and AGEs in all populations. A high AGE diet increased 8-isoprostanes in healthy adults, and vascular cell adhesion molecule-1 (VCAM-1) in patients with diabetes. Markers of CKD were not widely assessed. The evidence presented indicates that a high AGE diet may contribute to risk factors associated with chronic disease, such as inflammation and oxidative stress, however, due to a lack of high quality randomised trials, more research is required.

Keywords: systematic review; advanced glycation end-products; diet; chronic kidney disease; diabetes; cardiovascular disease; inflammation

1. Introduction

Lifestyle factors, such as diets high in fat, sugar and salt, play a key role in the development and progression of chronic diseases, such as type 2 diabetes (T2DM), cardiovascular disease (CVD) and chronic kidney disease (CKD) [1]. The modern Western diet is comprised of highly processed foods that are rich not only in fat, sugar and salt but also contain potentially pathogenic compounds known as advanced glycation end-products (AGEs). Two recent systematic reviews that examined the effect of dietary AGE consumption suggest a positive relationship between AGE intake and serum AGE levels, markers of inflammation, oxidative stress and insulin resistance [2,3]. However these reviews made conclusions based on animal, cohort, and cross-sectional studies rather than examining the true effect of dietary-AGEs on chronic disease markers through RCTs. Furthermore, biomarkers of renal function were not considered and as circulating AGEs are thought to be particularly toxic to the kidney [4–8]

it is of interest whether a high AGE diet may contribute to the development and progression of CKD, a major co-morbidity of other chronic diseases.

Current World Health Organisation dietary recommendations for the prevention of chronic disease recommend limiting the intake of free sugars, saturated fat and salt [9]. At present there are no recommendations surrounding the consumption of foods high in AGEs, such as heat-treated milk or cereals [10], which may be perceived as contributing to a healthy diet. In order to guide dietary recommendations for dietetic practice it is important to determine whether high AGE diets impact the development and progression of chronic conditions. Therefore, the objective of this systematic review was to determine the effect of high dietary AGE intake compared to a low AGE intake on biomarkers of systemic inflammation, oxidative stress and other risk factors for T2DM, CVD and CKD. Secondary outcomes of circulating and excreted AGE levels were also considered in order to examine associations between dietary AGE consumption, absorption and metabolism.

2. Materials and Methods

2.1. Eligibility Criteria

This systematic review was conducted in accordance with the preferred reporting items for systematic review and meta-analyses protocols (PRISMA-P) 2015 statement [11]. Studies were limited to randomised controlled trials (RCTs) [12] and performed in human adults (>18 years of age) in healthy or overweight populations, or in populations with obesity, type 1 diabetes (T1DM), T2DM or CKD. Interventions were included if they involved the consumption of a diet high in AGE content, Maillard reaction products (MRP) or Amadori compounds (precursors to AGE formation) compared with an isocaloric low AGE/MRP diet. A high AGE diet was defined as containing at least 30% more measured AGEs, MRP or Amadori compounds than the comparator or cooked using a method known to increase the formation of AGEs. Studies that investigated the AGE content of parenteral feeds were excluded as the AGEs bypass the gastrointestinal tract. Studies that only considered the postprandial response to single meals of varying AGE content were also excluded from this review as adverse consequences of dietary AGE consumption are thought to occur over longer time frames; a minimum time-frame of 1 week was required for inclusion. Other eligibility criteria included publication in English language, having undergone peer review and considering at least one of the outcomes of interest. Publications from any date were considered. The PICO (Patient/Population; Intervention; Comparator; Outcome) question addressed in this review was as follows: in healthy and diseased adults (P), does a high AGE diet (I) compared to a low AGE diet (C), effect biomarkers of inflammation, oxidative stress and risk factors for chronic disease (O)?

2.2. Information Sources

In total six databases were searched (SCOPUS, CINHAL, EMBASE, Medline, Biological Abstracts and Web of Science) in April 2015. The Cochrane library of clinical trials was also searched. The following keywords were used: (Concept 1) (advanced glycation end product OR carboxymethyllysine OR pentosidine OR maillard reaction product OR dicarbonyl OR carboxyethyllysine OR amadori product OR crosslink OR pyrraline OR methylglyoxal) AND (Concept 2) (diet OR food OR intake OR exogenous OR nutrition OR western diet OR processed food OR eat OR consumption OR bakery product OR animal derived NEAR fat or protein OR canned food OR heat treated near food OR diet). No limits in terms of language or years were set on databases when searching. Subject headings were also searched in order to locate relevant studies that may not contain key words in the title or abstract. Reference lists of relevant original research and reviews articles were hand searched.

2.3. Selection Process

Titles and abstracts of publications returned from the search were screened for relevance by one reviewer. Selected articles were retrieved and assessed for inclusion using the criteria described. Studies which could not be clearly included or excluded were screened by a second reviewer.

2.4. Data Management and Collection

Endnote was used to store and manage references for all studies returned for each database. Microsoft Excel was used to collate extracted data. All data were extracted by using a predefined data extraction template that was based on the National Health and Medical Research Council (NHMRC) of Australia data extraction template for RCTs and cohort studies.

2.5. Data Items

Data extraction variables included: affiliation/source of funding; study design (crossover/parallel); study duration; location/setting; population size and characteristics including inclusion and exclusion criteria; dietary intervention details such as method of delivery, differences in AGE content, energy content and macronutrient composition; method of assessing AGE content of diet; method of randomisation; blinding; measurement and reporting of compliance to dietary intervention; medication and supplementation; and any additional risks of bias identified.

2.6. Outcomes and Prioritisation

The primary outcomes of interest extracted from each study were differences in: biomarkers of inflammation (tumour necrosis factor alpha (TNFα), interleukin-6 (IL-6), C reactive protein (CRP) and monocyte chemoattractant protein-1 (MCP-1)); oxidative stress (8-isoprostane); T2DM (homeostatic model assessment insulin resistance (HOMA IR), fasting blood glucose (FBG), haemoglobin $A1_c$ ($HbA1_c$); CVD (oxidised low density lipoprotein (OxLDL)), vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1)); and CKD (urine albumin, serum creatinine, estimated glomerular filtration rate (eGFR) and plasma Cystatin C). Secondary outcomes of interest were levels of circulating, urinary and faecal carboxymethyl lysine (CML) a well-characterised AGE.

2.7. Risk of Bias and Quality Assessment

The Cochrane risk of bias tool [13] was used to assess the likelihood of bias at the study level for each of the included studies. The key domains of interest were: adequate sequence generation (method of randomisation); adequate allocation concealment (whether researchers or participants could foresee assignment); blinding (participant blinding and outcome assessor blinding considered separately); whether incomplete data was addressed; free of selective outcome reporting; and free of any other bias (transparent reporting of dietary intake, inclusion and exclusion criteria of participants, any baseline differences between groups). The overall risk of bias for each study was determined based on the total score (high, low, or unclear risk of bias) under each of the described domains. In addition, the American Dietetic Association (ADA) quality criteria checklist [14] was used to assess the quality of the included studies. The quality assessment checklist provides an overall quality measure of positive, negative, or neutral based on: relevance of the study; clarity of research question; unbiased selection of participants; whether study groups were comparable; unbiased and transparent handling of withdrawals; whether blinding was used; quality of reporting of the intervention; whether outcomes were clearly defined and the measurement of outcomes was valid and reliable, including appropriate nutrition measures; appropriate use of statistics; inclusion of a discussion of limitations of the study; and whether any bias exists related to funding source or conflict of interest. Two reviewers independently performed the quality assessment and any disagreements were discussed until consensus was reached.

2.8. Data Synthesis

Data for each of the primary and secondary outcomes were synthesised and where possible reported as means at baseline and end of the intervention for each study group. This data was used to calculate the effect size (Cohen's *d*) in order to compare the results across the studies. A Cohen's *d* of

0.2–0.3 was considered small; 0.5 medium; and 0.8 or greater as large. A negative sign indicates an adverse effect due to dietary intervention. For some studies it was not possible to calculate effect size due to pre and post-means or standard deviations not being reported.

3. Results

3.1. Studies Identified

A total of 5194 original articles were returned from the initial search (Figure 1). After screening of titles and abstracts, 43 full text articles were identified and retrieved for assessment. A total of 12 randomised controlled trials reported in 11 articles met eligibility criteria and were included in this review [15–25]. The total number of participants in the included studies was 293.

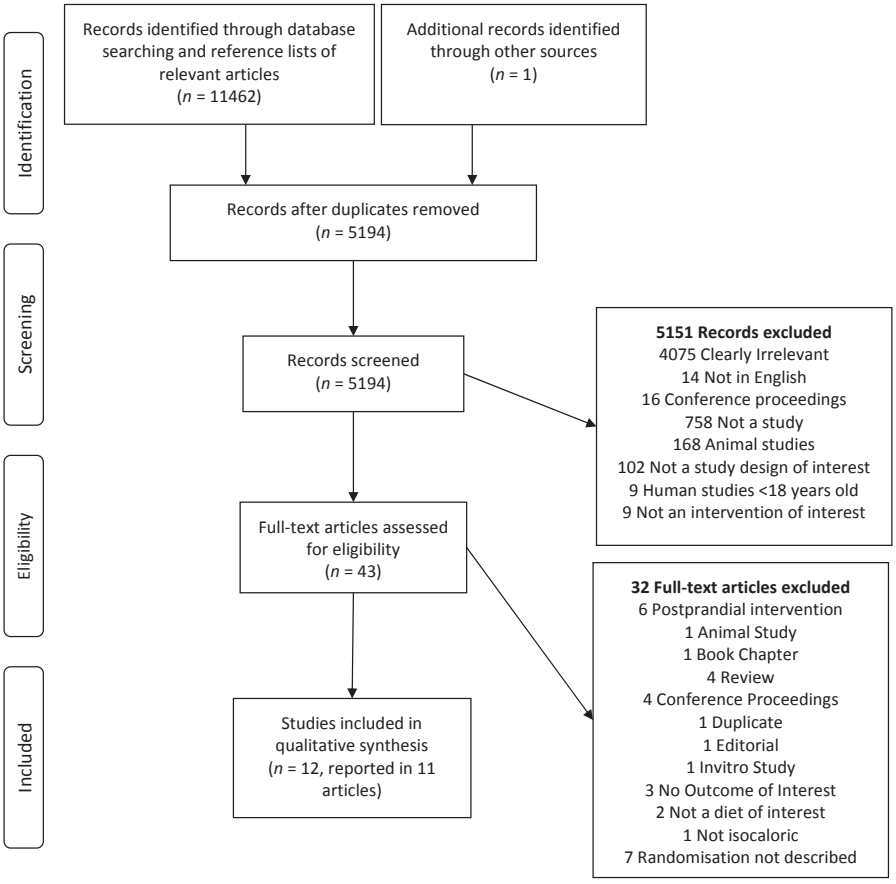

Figure 1. PRISMA Flow diagram of search results, screening and included studies.

3.2. Study Characteristics

The characteristics of included studies are shown in Table 1. All studies used cooking methods to generate differences in AGEs between diets. In six cases the dietary intervention was a high AGE diet [15,16,18,20,24] with a low AGE diet as a comparator. In the remaining six studies a standard diet high in AGEs was compared to an AGE restricted diet [17,19,21–23,25]. In several studies the standard

diets contained AGEs at levels similar to, or greater than, a high AGE diet used in another intervention, as measured by ELISA or estimated from an AGE database, and can therefore also be considered high AGE diets [15,19,22–25]. Participants were either provided study meals [15,16,18,20,24,25] or provided with instructions for meal preparation [17,19,21–23,25]. Dietary compliance was assessed by most studies using food diaries [16,17,19,21,23–25], a daily questionnaire [20], biweekly telephone calls [22], or a single telephone call over four-weeks [18]; however dietary compliance was not reported in one study [15]. Only one study [18] used liquid chromatography mass spectrometry (LC-MS) to measure the AGE content of the diets, three studies [15,24] used ELISA and eight studies [16,17,19–23,25] used the same reference database [26] to estimate AGEs in diets. Of the outcomes of interest considered in this review there were no studies that reported data for ICAM-1, eGFR, or faecal AGE content and only one study measured plasma cystatin C [16]. No studies reported any unexpected adverse outcomes due to the dietary interventions.

3.3. Studies in Healthy Populations

3.3.1. Biomarkers of Inflammation and Oxidative Stress

Three [21,22,25] studies in healthy individuals measured changes in circulating TNFα levels (Table 2). Of these, three [21,22,25] longer term studies (16-weeks) found that circulating TNFα was increased from baseline after consumption of the high AGE intervention compared with the low AGE intervention while the short-term study (two-weeks) did not observe differences between interventions [16]. The calculation of effect size [22] suggested a large negative effect on TNFα levels, or increase in this biomarker, due to the high AGE diet and a medium positive effect, or decrease, due to the low AGE diet [22].

Studies that measured circulating IL-6 [16,18] and CRP [16,20,21] levels following high AGE consumption from two to 16-weeks found no differences between interventions. Calculated effect sizes showed negligible effects on these biomarkers due to the high AGE diet [20] and a small but negative effect on CRP, indicating increased levels following the low AGE diet [20]. Only one study [16] measured MCP-1 and reported that there was an increase in this marker after only two-weeks of consumption of a high AGE diet compared with a low AGE diet in healthy overweight males. Three [21,22,25] studies reported increases in plasma levels of the oxidative stress marker 8-isoprostanes (a marker of lipid peroxidation) and one [16] observed an increase in urinary 8-isoprostanes after two-weeks consumption of a high AGE diet. Calculated effect size for one study [22] suggests the high AGE diet had a medium adverse effect on 8-isoprostanes levels, while the low AGE diet had a large positive effect in two studies [21,22].

3.3.2. Biomarkers of Chronic Disease Risk

In healthy adults, a high AGE diet did not increase risk factors for T2DM including fasting blood glucose [16,18,20,22,25] or $HbA1_c$ [22] (Table 3). One study reported an increase [18] in HOMA-IR after four-weeks on a high AGE diet while another long-term study observed no difference after 16-weeks [21] on a standard diet high in AGEs compared with a restricted AGE intake. The calculated effect sizes suggest a negligible or small effect due to the AGE content of the diet on T2DM risk factors including $HbA1_c$ and HOMA-IR, and a negligible or small effect of a low or reduced AGE diet [18,20,22]. Three studies [20,21,25] measured VCAM-1; two studies where food was provided to participants reported no significant differences between dietary interventions [20,25], while the other [21], in which participants prepared their own foods, found higher circulating levels of VCAM-1. The effect size calculated for this study revealed no effect due to the high AGE diet and a small positive effect, or reduction in VCAM-1, attributed to a low AGE diet [20]. Markers of kidney disease in healthy adults were assessed within two studies [16,25]. One short-term study in healthy overweight males reported increased urinary albumin-to-creatinine ratio and increased plasma cystatin C after two-weeks on a high AGE diet [16], whereas neither study observed differences in serum creatinine or creatinine clearance, respectively [16,25].

Table 1. Characteristics of included dietary interventions.

Study	Intervention	Comparator	Length (Weeks)	AGE Content of Diet	Assessment of AGEs	Participants Intervention	Comparator
Harcourt *et al.* 2011 [a] [16]	High AGE diet. Food provided. Cooking methods used to generate difference in AGEs. P:F:C = 16:30:54	Low AGE diet. Food provided. Cooking methods used to generate difference in AGEs. P:F:C = 16:30:54	2	H-AGE = 14,090, L-AGE = 3302 kU AGE/day	Based on reference database not validated [26]	Healthy overweight: n = 11 (M = 100%); Age = 30 ± 9 years; BMI = 31.8 ± 4.8 kg/m^2.	
Mark *et al.* 2014 [b] [18]	High AGE diet. Ingredients provided and participants instructed on how to prepare meals. Cooking methods used to generate difference in AGEs. P:F:C = 18.8 ± 0.4:37.3 ± 0.8:42.7 ± 0.9	Low AGE diet. Food provided. Cooking methods used to generate difference in AGEs. P:F:C = 21.6 ± 0.4:30.6 ± 0.7:46.9 ± 0.8	4	H-AGE = 24.6; L-AGE = 10.7 mg/day CML (H-AGE had 43% more AGEs)	LC-MS	Healthy overweight: n = 37 (M = 0); Age = 41.4 ± 1.4 years; BMI = 32.3 ± 0.6 kg/m^2	Healthy overweight: n = 36 (M = 0); Age = 37.9 ± 1.4 yrs; BMI = 33.2 ± 0.8 kg/m^2
Semba *et al.* 2014 [b] [20]	High AGE diet. Food provided. Cooking techniques and time used to produce AGEs. P:F:C = 17:29:55 *	Low AGE diet. Food provided. Cooking technique and time varied to reduce AGEs.	6	H-AGE = 4 times AGE content of L-AGE	Based on reference database not validated [26]	Healthy: n = 12 (M = 41.7%); Age = 60.6 ± 4.3 yrs; BMI = 26.1 ± 3.4 kg/m^2	Healthy: n = 12 (M = 41.7%); Age = 57.9 ± 6.0 yrs; BMI = 26.4 ± 4.0 kg/m^2
Uribarri *et al.* 2011 [b] [22]	Standard diet high in AGES. Participants prepared own food. Cooking techniques and time used to produce AGEs.	Low AGE diet. Participants prepared own foods under instruction. Cooking technique and time varied to reduce AGEs.	16	L-AGE = 40%–50% reduction in AGEs compared to H-AGE	Based on reference database not validated	Healthy: n = 9 (M = 22.2%); Age = 67 ± 1 years; BMI = 27.3 ± 1.4 kg/m^2	Healthy: n = 9 (M = 22.2%); Age = 67 ± 1 years; BMI = 27.3 ± 1.4 kg/m^2
Uribarri *et al.* 2014 [b] [21]	Standard diet high in AGES. Participants prepared own food. Cooking techniques and time used to produce AGEs.	Low AGE diet. Participants prepared own foods under instruction. Cooking technique and time varied to reduce AGEs.	16	H-AGE ⩾ 15; L-AGE < 10 kU AGE/day	Based on reference database not validated	Healthy: n = 8 (M = 25%); Age = 63.5 ± 5 years; BMI = 29 ± 2 kg/m^2	Healthy: n = 10 (M = 30%); Age = 65 ± 2 years; BMI = 26 ± 3 kg/m^2
Vlassara *et al.* 2009 [b] [25]	Standard diet high in AGES. Participants prepared own food. Cooking techniques and time used to produce AGEs.	Low AGE diet. Participants prepared own foods under instruction. Cooking technique and time varied to reduce AGEs.	16	H-AGE > 20,000; L-AGE < 10,000 kU CML/day	Based on reference database not validated [26]	30 Healthy participants randomised to either H-AGE or L-AGE diet. BMI = 28 ± 2 kg/m^2 Further population characteristics not described.	
Cai *et al.* 2004 [b] [15]	High AGE diet. Food provided. Cooking methods used to generate difference in AGEs. P:F:C = 20:30:50	Low AGE diet. Food provided. Cooking technique and time varied to reduce AGEs. P:F:C = 20:30:50	6	H-AGE = 16300 ± 3700; L-AGE = 3670 ± 1200 kU CML/day	Competitive ELISA for protein foods, direct ELISA for lipid foods. Not validated	T1DM and T2DM: n = 11 (M = 54%); Age = 61 ± 7 years; BMI = 28.2 ± 3.4 kg/m^2; T1DM:T2DM = 2:9	T1DM and T2DM n = 13 (M = 38%); Age = 62 ± 5 years; BMI = 28.7 ± 5.1 kg/m^2; T1DM:T2DM = 4:9
Luevano-Contreras *et al.* 2013 [b] [17]	Standard diet high in AGEs. Participants prepared own food. Cooking technique and time used to produce AGEs. P:F:C = 20:30:50	Low AGE diet. Participants prepared own foods under instruction. Cooking technique and time varied to reduce AGEs. P:F:C = 20:30:50	6	H-AGE = 9910 ± 4169; L-AGE = 8956 ± 3587 kU CML/day (from baseline)	Based on reference database not validated [26]	T2DM: n = 13 (M = 15.4%); Age = 48.5 ± 6.2 years; BMI = 28.8 ± 4. kg/m^2	T2DM: n = 13 (M = 7.7%); Age = 46.0 ± 5 years; BMI = 29.8 ± 4.0 kg/m^2
Uribarri *et al.* 2011 [b] [22]	Standard diet high in AGES. Cooking technique and time used to produce AGEs. Participants prepared own food.	Low AGE diet. Participants prepared own foods under instruction. Cooking technique and time varied to reduce AGEs.	16	L-AGE = 40%–50% reduction in AGEs compared to H-AGE	Based on reference database not validated	T2DM: n = 6 (M = 22.2%); Age = 61 ± 4 years; BMI = 32.3 ± 1.6 kg/m^2	T2DM: n = 12 (M = 22.2%); Age = 61 ± 4 years; BMI = 32.3 ± 1.6 kg/m^2
Vlassara *et al.* 2002 [b] [24]	High AGE diet. Food provided. Cooking techniques and time used to produce AGEs. P:F:C = 20:30:50	Low AGE diet. Food provided. Cooking technique and time varied to reduce AGEs. P:F:C = 20:30:50	6	H-AGE = 16,300 ± 3700; L-AGE = 3670 ± 1200 kU CML/day	Competitive ELISA for protein foods, direct ELISAs for lipid foods. Not validated	T1DM and T2DM: n = 6 Age = 62 years; BMI = 29.5 ± 3 kg/m^2 T1DM:T2DM = 4:8	T1DM and T2DM: n = 7 Age = 62 years; BMI = 29.5 ± 3 kg/m^2

Table 1. *Cont.*

Study	Intervention	Comparator	Length (Weeks)	AGE Content of Diet	Assessment of AGEs	Participants Intervention Comparator	
Vlassara *et al.* 2002 [a] [24]	High AGE diet. Food provided. Cooking methods used to generate difference in AGEs. P:F:C = 20:30:50	Low AGE diet. Cooking technique and time varied to reduce AGEs. P:F:C = 20:30:50	2	H-AGE = 16,300 ± 3700 L-AGE = 3670 ± 1200 kU CML/day	Competitive ELISA for protein foods, direct ELISAs for lipid foods. Not validated	T1DM and T2DM: n = 11 Age = 52 ± 5 years; BMI = 28 ± 3.5 kg/m^2 T1DM:T2DM = 2:9	
Uribarri 2003; Peppa 2004 [b] [19,23]	Standard diet high in AGEs. Participants prepared own food. Cooking techniques and time used to produce AGEs.	Low AGE diet. Cooking technique and time varied to reduce AGEs. Participants prepared own foods under instruction	4	H-AGE = 17,000 ± 3700; L-AGE = 5500 ± 900 kU CML/day	Based on reference database not validated [26]	Non diabetic peritoneal dialysis; n = 9 (M = 33.33%). Not significantly different from comparator at baseline.	Non diabetic peritoneal dialysis; n = 9 (M = 33.33%)
Vlassara *et al.* 2009 [b] [25]	Standard diet high in AGES. Participants prepared own food. Cooking techniques and time used to produce AGEs.	Low AGE diet. Meals prepared in the clinical research center and given to participants twice a week. Cooking technique and time varied to reduce AGEs.	4	H-AGE > 20,000; L-AGE < 10,000 kU CML/day	Based on reference database not validated [26]	9 CKD (stage 3) patients randomised to either H-AGE or L-AGE diet. BMI = 23 ± 1.6 kg/m^2 (intervention); 28 ± 1.9 kg/m^2 (comparator). Further population characteristics not described	

AGE = Advanced glycation end-product; BMI = body mass index; CML = carboxymethyl lysine; CKD = Chronic kidney disease; CRP = C-reactive protein; ELISA = Enzyme linked immunosorbent assay; FBG = fasting blood glucose; H-AGE = high AGE diet; $HbA1_c$ = glycated haemoglobin; HOMA IR = homeostatic model assessment insulin resistance; IL-6 = Interleukin 6; kU = kilo units; L-AGE = low AGE diet; LC-MS = Liquid chromatography- mass spectrometry; M = male; MCP-1 = monocyte chemoattractant protein-1; ND = Not described; P:F:C = percentage of total energy from protein, fat and carbohydrate of diet; PBMC = peripheral blood mononuclear cells; T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus; TNFα = tumour necrosis factor α; VCAM-1 = vascular cell adhesion molecule 1. Data reported as mean ± standard deviation; [a] randomised cross over trial; [b] randomised parallel arm trial; * Calculated from protein, fat and carbohydrate intake in grams/day.

Table 2. Circulating biomarkers of inflammation and oxidative stress.

Study	Group	*n*	TNFα	ES	IL-6	ES	CRP	ES	MCP-1	ES	8-Isoprostane	ES
Healthy												
Harcourt *et al.* 2011 [16]	L-AGE H-AGE	11CO			NVG		NVG		**H > L (plasma)**		**H > L (urine)**	
Semba *et al.* 2014 [20]	L-AGE	12			B: 1.48 (1.84) A: 1.53 (1.35) pg/mL	−0.03	B: 2.11 (1.45) A: 2.62 (2.25) mg/L	−0.27				
	H-AGE	12			B: 2.25 (1.84) A: 2.09 (1.35) pg/mL	+0.10	B: 1.57 (1.45) A: 1.38 (2.25) mg/L	+0.10				
Uribarri *et al.* 2011 [22]	L-AGE	9	**B: 10 (3.9) A: 8.4 (2.1) ng/mg (PBMC)**	+0.51							**B: 135 (30) A: 90 (27) pg/mL (plasma)**	+1.58
	H-AGE	9	**B: 8.6 (1.8) A: 11.8 (3) ng/mg (PBMC)**	−1.29							**B: 125 (54) A: 165 (69) pg/mL (plasma)**	−0.65
Uribarri *et al.* 2014 [21]	L-AGE	10	**MD: −2.1 (1.6) * ng/mg (PBMC)**				MD: 1.3 (1.4) * mg/L				**B: 170 (65) A: 85 (17) MD: −48 (11) * pg/mL (serum)**	+1.79
	H-AGE	8	**MD: +3.2 (0.8) * ng/mg (PBMC)**				MD: 0.4 (0.4) * mg/L				**MD: +48 (20) pg/mL (serum) ***	
Vlassara *et al.* 2009 [25]	L-AGE	30	**B: 12 (1) * A: 8 (0.5) * ng/mg (PBMC)**								B: 240 (67) * A: 100 (13) * ng/mL (plasma)	-
	H-AGE		**B: 9 (1) * A: 12 (1) * ng/mg (PBMC)**								B: 122 (4) * A: 173 (38) * ng/mL (plasma)	
Diabetes												
Luevano-Contreras *et al.* 2013 [17]	L-AGE	13	**MD: −18.36 (17.1) * pg/mL (serum)**				MD: −1.69 (5.4) * mg/L (serum)					
	H-AGE	13	**MD: +12.5 (14.7) * pg/mL (serum)**				MD: −1.21 (5.5) * mg/L (serum)					
Uribarri *et al.* 2011 [22]	L-AGE	12	**B: 18 (3.5) A: 14.4 (6.9) ng/mg (PBMC)**	+0.66							B: 233 (58.9) A: 141 (62.4) pg/mL (plasma)	+1.52
	H-AGE	6	**B: 20 (4.9) A: 26 (4.9) ng/mg (PBMC)**	−1.22							B: 236 (61.2) A: 313 (188.6) pg/mL (plasma)	−0.55

Table 2. *Cont.*

Study	Group	*n*	TNFα	ES	IL-6	ES	CRP	ES	MCP-1	ES	8-Isoprostane	ES
Healthy												
Vlassara *et al.* 2002 6-weeks [24]	L-AGE	7	**MD:−20% * ng/mL (PBMC)**				**MD: −20% * mg/dL (serum)**					
	H-AGE	6	**MD: +86.3% * ng/mL (PBMC)**				**MD: +35% * mg/dL (serum)**					
Vlassara *et al.* 2002 2-weeks [24]	L-AGE	11CO					MD: 4.1 (4.8) * mg/dL (serum)					
	H-AGE						MD: 6 (8.6) * mg/dL (serum)					
Kidney Disease												
Uribarri *et al.* 2003; Peppa 2004 *et al.* [19,23]	L-AGE	9	**B: 44 (18) A: 31 (10.8) pg/mg (PBMC)**	+0.88			**Graph only**					
	H-AGE	9	**B: 43 (21) A: 44 (21) pg/mg (PBMC)**	−0.05			Graph only					
Vlassara *et al.* 2009 [25]	L-AGE	9	**B: 22 (4) * A: 16 (3) * ng/mg (PBMC)**								**B: 328 (51) * A: 154 (25) * ng/mL (plasma)**	
	H-AGE		B: 15 (4) * A: 16 (3) * ng/mg (PBMC)								B: 211 (7) * A: 167 (21) * ng/mL (plasma)	

Abbreviations: A = after intervention; AGE = Advanced glycation end-product; B = baseline; CO = Crossover trial; CRP = C reactive protein; ES = Effect Size, H-AGE = high AGE diet; IL-6 = Interleukin-6; L-AGE = low AGE diet; MCP-1 = monocyte chemoattractant protein-1; MD = mean difference from baseline; NVG = no value give; PBMC = peripheral blood mononuclear cells; TNFα = tumour necrosis factor α. Data reported as mean (standard deviation) except where * indicates mean (standard error of the mean). Bold indicates significant differences between groups or from baseline ($p < 0.05$). Bold indicates significant differences between groups or from baseline ($p < 0.05$).

Table 3. Biomarkers of chronic disease risk.

Study	Group	*n*	T2DM				CVD						CKD			
			HOMA IR	ES	FBG	ES	$HbA1_c$ %	ES	oxLDL	ES	VCAM-1	ES	Alb	ES	Cr	ES
Healthy																
Harcourt *et al.* 2011 [16]	L-AGE	11CO			A: 5.1 (0.3) mmol/L								**H > L**		A: 72.3 (18.3) µmol/L (serum)	
	H-AGE															
Mark *et al.* 2014 [18]	L-AGE	36	**B: 2.65 (1.8) A: 2.43 (1.8)**	+0.12	B: 5.4 (0.6) A: 5.5 (0.6) mmol/L	−0.17										
	H-AGE	37	**B: 2.14 (1.8) A: 2.40 (1.2)**	−0.17	B: 5.5 (0.61) A: 5.5 (0.61) mmol/L	0										
Semba *et al.* 2014 [20]	L-AGE	12			B: 97 (10.4) A: 93 (10.4) mg/dL	+0.38					B: 1.26 (0.8) A: 1.01 (0.9) ug/mL	+0.29				
	H-AGE	12			B: 93 (10.4) A: 96 (10.4) mg/dL	−0.29					B: 1.39 (0.8) A: 1.34 (0.9) ug/mL	−0.06				
Uribarri *et al.* 2011 [22]	L-AGE	9	B: 2.2 (0.9) A: 2.5 (1.8)	−0.33	B: 86 (9) A: 88 (18) mg/dL	−0.14										
	H-AGE	9	B: 1.3 (0.9) A: 1.5 (1.8)	−0.22	B: 83 (12) A: 80 (12) mg/dL	+0.25										
Uribarri *et al.* 2014 [21]	L-AGE	10	MD: 0.04 (0.44) *								**MD: −270 (92) * pg/mL (serum)**					
	H-AGE	8	MD: 0.14 (0.15) *								**MD:+182 (46) * pg/mL (serum)**					
Vlassara *et al.* 2009 [25]	L-AGE	30			B: 81 (3) * A: 78 (3) * mg/dL						B: 1001 (115) * A: 576 (49) * ng/mL (plasma)				B: 99 (12) * A: 118 (15) * mL/min (Cr clearance)	
	H-AGE				B: 84 (3) * A: 79 (3) * mg/dL						B: 717 (68) * A: 771 (58) * ng/mL (plasma)				B: 115 (10) * A: 100 (8) * mL/min (Cr clearance)	

Table 3. *Cont.*

Study	Group	*n*	T2DM						CVD				CKD			
			HOMA IR	ES	FBG	ES	$HbA1_c$ %	ES	oxLDL	ES	VCAM-1	ES	Alb	ES	Cr	ES
Diabetes																
Cai *et al.* 2004 [15]	L-AGE	13			B: 122 (90.1) A: 118 (68.5) mg/dL	+0.05	B: 7.2 (3.6) A: 7.0 (2.88)	+0.06	**A:1.5 (0.5) nmol/mg**							
	H-AGE	11			B: 116 (146.6) A:128 (71.6) mg/dL	−0.10	B: 7.3 (2.0) A: 7.4 (4.3)	−0.03	**A:5.7 (2.3) nmol/mg**							
Luevano-Contreras *et al.* 2013 [17]	L-AGE	13	MD: −2.29 (3.7) *		MD: −18 (56.7) * mg/dL		MD: 0.19 (1.3) *									
	H-AGE	13	MD: −2.5 (6.1)*		MD: 4.55 (35.6) * mg/dL		MD: −0.11 (1.9) *									
Uribarri *et al.* 2011 [22]	L-AGE	12	**B: 5.3 (1.4) A: 3.4 (2.1)**	+1.06	B: 114 (24.2) A: 111 (31.2) mg/dL	+0.11	B: 6.4 (0.7) A: 6.6 (1.4)	−0.18								
	H-AGE	6	**B: 4.5 (2.9) A: 6.2 (1.2)**	−0.77	B: 131 (90) A: 129 (63.7) mg/dL	+0.03	B: 6.7 (1.2) A: 6.5 (1.0)	+0.18								
Vlassara *et al.* 2002 6-weeks [24]	L-AGE	7			B: 7.0 (2.7) A: 5.6 (1.3) mmol/L	+0.68					MD: −20% * ng/mL (serum)					
	H-AGE	6			B: 6.5 (2.9) A: 8.1 (2.7) mmol/L	−0.57										
Vlassara *et al.* 2002 2-weeks [24]	L-AGE	11CO			**A: 7.5 (0.7) * mmol/L**						**MD:698 (347) * ng/mL (serum)**					
	H-AGE				**A: 8.1 (0.4) * mmol/L**						**MD:1108 (429) * ng/mL (serum)**					

Table 3. *Cont.*

Study	Group	*n*	T2DM						CVD				CKD	
			HOMA IR	ES	FBG	ES	$HbA1_c$ %	ES	oxLDL	ES	VCAM-1	ES	Alb	ES	Cr	ES
Kidney Disease																
Uribarri *et al.* 2003; Peppa 2004 *et al.* [19,23]	L-AGE	9									**B: 3448 (483) A: 3244 (708) ng/mL (serum)**	+0.34				
	H-AGE	9									**B: 3699 (306) A: 3735 (291) ng/mL (serum)**	−0.12				
Vlassara *et al.* 2009 [25]	L-AGE	9			B: 89 (3) * A: 90 (3) * mg/dL						B: 1033 (168) * A: 733 (52) * (ng/mL) (plasma)				B: 39.5 (11) * A: 36 (10) * mL/min (Cr clearance)	
	H-AGE				B: 93 (2) * A: 80 (4) * mg/dL						B: 1086 (210) * A: 956 (133) * (ng/mL) (plasma)				B: 46.5 (15) * A: 43 (11) * mL/min (Cr clearance)	

Abbreviations: A = after intervention; AGE = Advanced glycation end-product; Alb = Albuminuria; B = baseline; CO = Crossover trial; Cr = creatinine; ES = Effect Size; FBG = fasting blood glucose; H-AGE = High AGE diet; $HbA1_c$ = glycated haemoglobin; HOMA IR = homeostatic model assessment insulin resistance; L-AGE = Low AGE diet; MD = mean difference from baseline; NS = not significant; oxLDL = oxidised low density lipoprotein; VCAM-1 = vascular cell adhesion molecule 1. Data are reported mean (standard deviation) except where * indicates mean (standard error of the mean). Bold indicates significant differences between groups ($p < 0.05$). Data reported with significant figures as described in publication. Bold indicates significant differences between groups or from baseline ($p < 0.05$).

3.3.3. Circulating and Excreted CML

The effect of a high AGE diet on circulating CML varied with study duration (Table 4). Three long-term trials [21,22,25] in healthy adults reported an increase in circulating CML 16-weeks after consumption of a high AGE diet or standard diet high in AGEs compared with an AGE restricted diet. Calculated effect sizes indicate a large positive, or lowering, effect of a low AGE diet on serum AGEs across studies of varying durations (four to 16-weeks) [20–22]; the effect of a high AGE diet on serum AGEs is less clear with a small positive effect seen in one short-term study (four-weeks) and a medium negative, or increasing effect seen in a longer term study (16-weeks) [22]. A two-week crossover trial [16] in healthy overweight males found circulating AGEs reduced after the high AGE intervention compared with a restricted AGE diet whilst urinary AGE excretion increased. Only one other study [20] measured urinary CML in healthy adults and reported no differences between diets; similarly the calculated effect sizes were negligible due to both diets.

3.4. Studies in Patients with Diabetes

3.4.1. Biomarkers of Inflammation and Oxidative Stress

Similar to the effects observed in healthy participants, studies in individuals with diabetes that measured TNFα found that the high AGE intervention resulted in significantly higher circulating levels [17,22,24] (Table 2). For one study it was possible to calculate the effect size [22] which demonstrated a large negative effect due to high AGE intervention and a medium positive effect of the low AGE diet on TNFα levels. Of the studies that measured CRP levels, two [17,24] reported no differences due to intervention while one [24] demonstrated that CRP increased after six-weeks on a high AGE diet. The only study [22] that measured 8-isoprostanes as a marker of oxidative stress in T2DM patients reported significantly higher mean plasma concentrations after 16-weeks on a standard diet high in AGEs. The effect size calculated for this study suggests that the adverse effect of the high AGE diet on 8-isoprostanes was moderate, while the low AGE diet was seen to have a large positive effect [22] (Table 2).

3.4.2. Biomarkers of Chronic Disease Risk

The studies that examined the effect of a high AGE diet on insulin resistance in patients with diabetes were conflicting [17,22] (Table 3). The calculated effect size for one study suggested that the high AGE diet had a moderate negative effect on, or increased, HOMA-IR while the low-AGE diet had a large positive effect [22]. The majority of the included articles suggested that a high AGE diet has no effect on fasting blood glucose levels [15,17,22,24] or $HbA1_c$ [22] in this population. CVD risk factors were reported to increase in patients with diabetes on a high AGE diet, though it was not possible to determine effect size for any study [15,24]. The one study that measured oxidised LDL observed increased plasma levels following six-weeks on a high AGE diet [15]. Standard diets high in AGEs resulted in increased levels of VCAM-1 when compared to a reduced AGE intervention after two-weeks and six-weeks of consumption [24].

3.4.3. Circulating and Excreted CML

Like healthy populations, circulating CML was increased due to a high AGE diet in the majority of studies [15,22,24] in patients with diabetes (Table 4). Calculated effect sizes showed medium to large negative effects on, or increases in circulating AGES due to the high AGE diet and medium to large positive effects, or decreases due to the low AGE diet [19,22]. Urinary CML was measured in only one two-week crossover trial and was reported to be higher following consumption of the high AGE diet suggesting increased excretion [24].

Table 4. Circulating levels and urinary excretion of the advanced glycation end-product Carboxymethyl Lysine (CML).

Study	Group	*n*	Circulating CML	ES	Urinary CML	ES
Healthy						
Harcourt *et al.* 2011 [16]	L-AGE H-AGE	11CO	**L > H**		**H > L**	
Mark *et al.* 2014 [18]	L-AGE H-AGE	36 37			**H > L**	
Semba *et al.* 2014 [20]	L-AGE	12	B: 763 (83) A: 678 (100) ng/mL (serum)	+0.92	B: 1.37 (5.10) A: 0.77 (6.96) µg/mg creatinine	+0.10
	H-AGE	12	B: 751 (83) A: 711 (100) ng/mL (serum)	+0.44	B: 1.03 (5.10) A: 1.21 (6.96) µg/mg creatinine	−0.03
Uribarri *et al.* 2011 [22]	L-AGE	9	**B: 12.4 (1.5)** **A: 9.3 (3.0) U/mL (serum)**	+1.31		
	H-AGE	9	**B: 11.7 (3.9)** **A: 14.0 (3.0) U/mL (serum)**	-0.66		
Uribarri *et al.* 2014 [21]	L-AGE	10	**B: 13.7 (3.2) A: 9.2 (2.5)** **MD: −3.71 (1.03) * U/mL (serum)**	+1.57		
	H-AGE	8	**MD: +1.87 (1.05) * U/mL serum)**	-		
Vlassara *et al.* 2009 [25]	L-AGE	30	**B: 14 (1) *** **A: 9 (1) * U/mL (serum)**	-		
	H-AGE		**B: 11 (1) *** **A: 13 (1) * U/mL (serum)**			
Diabetes						
Cai *et al.* 2004 [15]	L-AGE	13	**B: 12.5 (7.9)** **A: 7.9 (4.0) U/mL (serum)**	+0.73		
	H-AGE	11	**B: 13.1 (8.6)** **A: 18.0 (5.6) U/mL (serum)**	−0.68		
Uribarri *et al.* 2011 [22]	L-AGE	12	**B: 17.1 (4.5)** **A: 11.6 (3.8) U/mL (serum)**	+1.32		
	H-AGE	6	**B: 17.8 (4.9)** **A: 24.2 (9.8) U/mL (serum)**	−0.83		

Table 4. *Cont.*

Study	Group	*n*	Circulating CML	ES	Urinary CML	ES
Vlassara *et al.* 2002 6-weeks [24]	L-AGE	7	**MD: −40% U/mL (serum)**			
	H-AGE	6	**MD: 28.2 % U/mL (serum)**			
Vlassara *et al.* 2002 2-weeks [24]	L-AGE	11CO	**A: 7.7 (2.4) * U/mL (serum)**		**A: 15.26 (10) $\times$ 10^{-3} U/24 h**	
	H-AGE		**A: 13 (6) * U/mL (serum)**		**A: 30.4 (12) $\times$ 10^{-3} U/24 h**	
Kidney Disease						
Uribarri *et al.* 2003; Peppa 2004 *et al.* [19,23]	L-AGE	9	**MD: −34% (serum)**			
	H-AGE	9	**MD: 29% (serum)**			
Vlassara *et al.* 2009 [25]	L-AGE	9	**B: 25 (3) * A: 14.2 (2) * U/mL (serum)**			
	H-AGE		**B: 19 (4) * A: 17 (3) * U/mL (serum)**			

Abbreviations: A = after intervention; AGE = Advanced glycation end-product; B = baseline; CML = carboxymethyl lysine; CO = crossover trial; ES = Effect Size; H-AGE = high AGE diet; L-AGE = low AGE diet; MD = mean difference from baseline; N/A = Not assessed; U = units. Data reported as mean (standard deviation) except where * indicates mean (standard error of the mean). − = insufficient data to calculate effect size. Bold indicates significant differences between groups ($p < 0.05$). Data reported with significant figures as described in publications. Bold indicates significant differences between groups or from baseline ($p < 0.05$).

3.5. Studies in Patients with CKD

3.5.1. Biomarkers of Inflammation and Oxidative Stress

Consistent with studies in healthy individuals and patients with diabetes, studies in patients with CKD measured TNFα and reported an increase due to a high AGE diet [19,23,25] (Table 2). Effect size could be calculated for only one study [19,23] where the effect of the high AGE diet on TNFα was negligible; while the low AGE diet was found to have a large positive effect [19,23]. Levels of CRP were assessed in one study [19,23] and reported to significantly decrease following the low AGE diet though statistical differences between groups were not described. One study reported a greater decrease in circulating 8-isoprostanes after 4-weeks on a low AGE diet compared with a high AGE [25].

3.5.2. Biomarkers of Chronic Disease Risk

Markers of T2DM risk were not widely assessed in patients with CKD (Table 3). Only one study measured fasting blood glucose and reported no change after four-weeks of dietary intervention [25]. It is not clear whether a high AGE diet increases CVD risk in CKD patients. One study observed an increase in levels of VCAM-1 due to the high AGE diet [19,23] while the other reported no effect [25]. Calculated effect sizes due to high AGE diet were negligible while the low AGE diet had a small positive effect, meaning a reduction in VCAM-1 levels [19,23]. In addition, the effect of the dietary interventions on biomarkers of kidney disease was not clear. Urine albumin and plasma Cystatin C were not reported in either study, while serum creatinine was reported in one study with no differences observed [25].

3.5.3. Circulating and Excreted CML

Again, similar to the studies in other populations, circulating CML was higher in both studies in patients with kidney disease following consumption of a high AGE diet compared to a low AGE intake [19,23,25] (Table 4).

3.6. Quality Assessment

The results of the risk of bias assessment and quality assessment are shown in Table 5. In all studies it was not possible to blind the participants to their allocation group as cooking methods were used to produce a difference in AGE content in the diet. As knowledge of treatment group would be unlikely to introduce bias into the outcomes measured, the studies were evaluated based only on the blinding of outcome assessors. One study received a low risk of bias score as defined by a low risk of bias in all key domains [20]. Sources of bias included: not reporting dietary intake [15,21,22,25]; not reporting smoking status [19,22,23,25]; not reporting baseline characteristics or assessing differences between groups at baseline [19,22–25]; known differences in groups at baseline [17] possible confounding due to weight loss during study [18]; and supplementing both diet groups with beverages high in either fructose or glucose [18].

The quality of the methods employed while conducting the dietary trial was assessed with the quality assessment tool as previously described. Three studies [19,23,25] were found to be of poor quality, including both trials in patients with CKD, due to potential confounding from smoking status not being reported or compliance to dietary intervention not being adequately assessed. The remainder were given a neutral score with not reporting method of randomisation a reoccurring flaw. A summary of overall findings, risk of bias, and quality score is provided in Table 6.

Table 5. Assessment of studies using Cochrane risk of bias tool and the American Dietetic Association quality criteria checklist.

	Adequate Sequence Generation	Adequate Allocation Concealment	Blinding-Outcome Assessors	Incomplete Outcome Data Addressed	Free of Selective Outcome Reporting	Free of Other Bias	Overall Risk of Bias	Quality (+, −, or Neutral)
Healthy								
Harcourt *et al.* 2011 [16]	Unclear	Unclear	Unclear	+	Unclear	+	Unclear	Neutral
Mark *et al.* 2014 [18]	Unclear	Unclear	-	+	Unclear	–(weight loss occurred and supplemented with high fructose or glucose beverages)	High	Neutral
Semba *et al.* 2014 [20]	+	+	+	+	+	+	Low	Neutral
Uribarri *et al.* 2011 [22]	Unclear	Unclear	Unclear	+	Unclear	Unclear (differences in intervention groups at baseline, smoking and dietary intakes not reported)	Unclear	Neutral
Uribarri *et al.* 2014 [21]	Unclear	Unclear	Unclear	+	+	Unclear (dietary intake not reported)	Unclear	Neutral
Vlassara *et al.* 2009 [25]	Unclear	Unclear	Unclear	+	Unclear	Unclear (differences in intervention groups at baseline, smoking and dietary intakes not reported)	Unclear	-
Diabetes								
Cai *et al.* 2004 [15]	Unclear	Unclear	Unclear	+	Unclear	Unclear (dietary intake not reported)	Unclear	Neutral
Luevano-Contreras *et al.* 2013 [17]	+	+	+	+	Unclear	–(difference in TNFα levels between groups at baseline)	High	Neutral
Vlassara *et al.* 2002 2-weeks [24]	Unclear	Unclear	Unclear	+	-	Unclear (baseline characteristics not reported in detail)	High	Neutral
Vlassara *et al.* 2002 6-weeks [24]	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear (baseline characteristics not reported)	Unclear	Neutral

Table 5. *Cont.*

	Adequate Sequence Generation	Adequate Allocation Concealment	Blinding-Outcome Assessors	Incomplete Outcome Data Addressed	Free of Selective Outcome Reporting	Free of Other Bias	Overall Risk of Bias	Quality (+, −, or Neutral)
Kidney Disease								
Uribarri *et al.* 2003/Peppa *et al.* 2004 [19,23]	Unclear	Unclear	Unclear	+	Unclear	Unclear (intervention group significantly increased caloric intake from baseline, smoking not reported)	Unclear	-
Vlassara *et al.* 2009 [25]	Unclear	Unclear	Unclear	+	Unclear	Unclear (differences in intervention groups at baseline, smoking and dietary intakes not reported)	Unclear	-

Abbreviations: TNFα = Tumour necrosis factor alpha. + = Yes/ Free of bias/high quality; − = No/risk of bias/poor quality.

Table 6. Summary of findings from included studies.

Study	Length (weeks)	Population	Inflammation	Oxidative Stress	T2DM Risk	CVD Risk	CKD Risk	cAGEs	uAGEs	Risk of Bias	Quality
Healthy											
Harcourt *et al.* 2011 [16]	2	Healthy overweight	↑/↔	↑	↔	N/A	↑/↔	↓	↑	Unclear	Neutral
Mark *et al.* 2014 [18]	4	Healthy overweight	N/A	N/A	↑/↔	N/A	N/A	N/A	↑	High	Neutral
Semba *et al.* 2014 [20]	6	Healthy	↔	N/A	↔	↔	N/A	↔	↔	Low	Neutral
Uribarri *et al.* 2011 [22]	16	Healthy	↑	↑	↔	N/A	N/A	↑	N/A	Unclear	Neutral
Uribarri *et al.* 2014 [21]	16	Healthy	↑/↔	↑	↔	↑	N/A	↑	N/A	Unclear	Neutral
Vlassara *et al.* 2009 [25]	16	Healthy	↑	↔	↔	↔	N/A	↑	N/A	Unclear	-
Diabetes											
Cai *et al.* 2004 [15]	6	T1DM + T2DM	N/A	N/A	↔	↑	N/A	↑	N/A	Unclear	Neutral
Luevano-Contreras *et al.* 2013 [17]	6	T2DM	↑/↔	N/A	↔	N/A	N/A	↔	N/A	High	Neutral
Uribarri *et al.* 2011 [22]	16	T2DM	↑	↑	↑/↔	N/A	N/A	↑	N/A	Unclear	Neutral
Vlassara *et al.* 2002 [24]	6	T1DM + T2DM	↑	N/A	↑	↑	N/A	↑	↑	High	Neutral
Vlassara *et al.* 2002 [24]	2	T1DM + T2DM	↔	N/A	↑	↑	N/A	↑	N/A	Unclear	Neutral
Kidney Disease											
Uribarri *et al.* 2003; Peppa 2004 [19,23]	4	CKD	↑	N/A	N/A	↑	N/A	↑	N/A	Unclear	-
Vlassara *et al.* 2009 [25]	4	CKD	N/A	↑	↔	↔	↔	↑	N/A	Unclear	-

Abbreviations: AGE = Advanced glycation end-product; cAGEs = circulating AGEs; CKD = Chronic kidney disease; CVD = cardiovascular disease; N/A = Not assessed; T2DM = type 2 diabetes mellitus; uAGEs = urinary AGEs. ↑ = significantly increased following a high AGE diet or standard diet high in AGEs when compared to a low AGE diet; ↔ = Negligible effect size and/or no differences in outcome between a high AGE and low AGE diet; ↓ = significantly decreased; ↓ significantly decreased following a high AGE diet or standard diet high in AGEs when compared to a low AGE diet.

4. Discussion

4.1. The Effect of a High AGE Diet

This is the first systematic review of RCTs to examine the effect of a high AGE diet on biomarkers of inflammation, oxidative stress and chronic disease risk factors in humans. Unlike previous reviews, this study also considers the effect of a high AGE diet on risk factors for CKD; a highly important consideration given that dietary AGEs may be potentially toxic to the kidneys in individuals susceptible to CKD. The studies presented here used cooking methods to generate differences in the AGE content of interventions, at levels comparable to those found in the Western diet, and so can be considered physiologically relevant. Overall the evidence indicates that consumption of a high AGE diet increases pro-inflammatory biomarkers, specifically TNFα, and circulating levels of AGEs in healthy and overweight individuals and patients with T1DM, T2DM or CKD. The studies reviewed here also suggest that consumption of a high AGE diet may increase biomarkers of oxidative stress in healthy adults, increase cardiovascular risk factors in patients with diabetes, and promote renal dysfunction in overweight males. However, as various methodological issues were identified with several of the studies and high heterogeneity observed between the studies; the results presented here should be interpreted with caution.

There was a lack of evidence surrounding the effect of a high AGE diet on biomarkers of CKD. This is surprising considering the number of studies in patients with CKD [19,23,25] or with diabetes [15,17,22,24] which is a major cause of end-stage renal disease. Overweight and obese, but otherwise healthy individuals are at an increased risk of developing CKD [27]. Therefore, the fact that short-term exposure to a high AGE diet resulted in albuminuria is highly significant. Further studies investigating the effect of a high AGE diet on renal function in healthy populations and individuals with established chronic disease are warranted.

Previous reviews suggest that low AGE intake may be beneficial in reducing biomarkers of inflammation [2,3]. In the current review, the consumption of a high AGE diet for periods greater than two-weeks appeared to increase circulating TNFα levels. This is consistent with studies in cells and in animals which have shown that AGE interaction with the receptor for advanced glycation end-products (RAGE) leads to prolonged activation of nuclear factor kappa B (NFκB) which results in the transcription of pro-inflammatory cytokines, including TNFα [28,29]. Despite an increase in TNFα levels with AGE intake, the same trend was not observed in circulating IL-6 levels. However, IL-6 is known to function as a pro-inflammatory or an anti-inflammatory cytokine depending on the signalling cascade which is activated [30], making these result difficult to interpret. Similarly, the majority of studies presented here also observed no difference in CRP levels. Whilst MCP-1 was measured in in only one short-term study and was increased in response to consumption of a high AGE diet [16]. It is possible that restricting AGE intake could be beneficial in reducing levels of some inflammatory markers, such as TNFα, which could help to prevent or slow the progression of chronic conditions, such as insulin resistance or atherosclerosis [31] however further high quality evidence is required.

Patients with T2DM are thought to have increased levels of oxidative stress, in particular, of circulating 8-isoprostanes, a marker of lipid peroxidation [32]. Hyperglycaemia induces the generation of reactive oxygen species (ROS) [33,34], promoting the production of 8-isoprostanes through the peroxidation of arachidonic acid [32]. The effect of a high AGE diet on oxidative stress in individuals with diabetes was not well characterised in the included studies. This report suggests that restricting dietary AGE intake in patients with diabetes may be beneficial in reducing levels of oxidative stress. Analysis of the results for the studies involving healthy cohorts [21,22,25] demonstrated that the restricted AGE diet reduced 8-isoprostanes levels, which is in agreement with the findings of an earlier systematic review [2]. Similar to individuals with diabetes, patients with CKD have also been observed to have higher levels of 8-isoprostanes [32] possibly due to impaired renal clearance. Low quality evidence was found which suggests that an AGE restricted diet may lower plasma 8-isoprostanes in

patients with CKD. However, only a small number of participants (n = 9) were included and therefore, the generalisability of these results are limited. In addition, the authors did not specify whether these patients suffered from comorbidities such as diabetes, which may have confounded results. Again, further higher-quality studies with larger samples sizes are required before a conclusion can be made about the association between a high AGE diet and oxidative stress in chronic conditions.

All of the studies that reported significant increases in circulating AGEs (measured as CML) in response to a high AGE diet were performed within the same research group [19,21–25]. Before it can be confirmed whether dietary AGEs contribute to elevated plasma levels of AGEs further high quality randomized trials need to be performed by independent research groups. It should be highlighted that there may have been overlap in participants between two 6-week parallel arm intervention studies from the same group of investigators [15,24]. As there were differences in the baseline characteristics reported between articles, the participant groups were treated separately. This may have led to the exaggeration of some of the results obtained in this review.

Six studies across all populations observed parallel changes in serum AGEs and inflammatory and/or oxidative stress markers [19,21–25], which suggests that AGEs from heat-treated and processed foods are absorbed and enter the circulation where they can drive systemic inflammation. However, two studies reviewed here reported increased levels of pro-inflammatory markers despite not seeing an increase in serum AGEs [16,17]. This gives rise to the idea that a high AGE diet may increase inflammation through mechanisms other than direct absorption into the circulation, such as via effects on gut homeostasis. Few studies in humans have looked at the metabolic fate of dietary AGEs and no studies included in this review measured the AGE content of faecal samples. Recent studies in adolescents suggest that AGEs and other Maillard reaction products may disrupt the composition of the gut microbiota [35]. As changes in microbiota composition are associated with increases in systemic inflammation [36,37] it is possible that the dietary AGEs which escape absorption may also trigger inflammation via this mechanism. Future research in this area should aim to include detailed measurement of the fate of AGEs *in vivo* (*i.e.*, urinary and faecal AGE concentrations or changes to microbiota composition) in order to delineate the physiological mechanisms by which dietary AGEs might elicit their effects.

Other than inflammatory factors, the studies performed to date suggest that a high AGE diet does not increase risk factors for T2DM in healthy or overweight individuals, patients with diabetes or patients with CKD. Dietary AGEs do not influence fasting blood glucose levels or $HbA1_c$ [15–17,20–22,24,25], however animal studies indicate that dietary AGEs may target pancreatic islets impairing the function of insulin secreting beta cells [38–40]. An earlier systematic review [2] reported that there was low-quality evidence [22,41] to indicate that a low AGE diet is beneficial for improving insulin sensitivity in patients with diabetes, yet recent research included in this review does not support this idea [17]. The only short term study [18] that reported reduced insulin sensitivity (HOMA-IR) in healthy women had a high risk of bias and possible confounding due to differences in dietary fat intake between the interventions and weight loss between groups; therefore the impact on insulin sensitivity cannot be conclusively attributed to dietary AGE intake in this study. Over longer periods of AGE consumption [21,22], it appears that it is unlikely that dietary AGEs influence insulin sensitivity, especially in individuals free of chronic disease.

There is evidence to suggest high AGE diets promote risk factors for CVD in patients with diabetes [15,24]. VCAM-1 is an early marker of unstable atherosclerotic plaques [42] and OxLDL is significantly associated with cardiovascular events and stroke in humans [43]. VCAM-1 was improved in response to a low AGE diet [24], while OxLDL was increased following consumption of a high AGE diet in patients with diabetes [15]. This is an important finding as patients with diabetes are at an increased risk of CVD. It appears that reduction of dietary AGEs through simple modification of cooking methods, even in the absence of caloric restriction, may actively reduce CVD risk in these patients. However all studies that measured CVD risk outcomes in this review were from the same research team and before any recommendations can be made these results need to be further verified.

4.2. Limitations of Included Studies

The majority of studies included in this review scored low in the quality analysis due to gaps in reporting and methodological flaws, thus limiting the strength of the findings from this review. Only one study reported a power calculation [18] although still failed to achieve the required number of participants to see a significant change in the primary outcome of interest (HOMA-IR) due to drop-outs. As this study had the largest sample size of any study reviewed here, it is therefore highly likely that most studies were underpowered. Executing controlled dietary studies with free-living participants is incredibly difficult due to economic constraints, and burden on the participants, and these factors likely contributed to the small sample sizes in the included studies.

The diversity of study populations further complicated the interpretation of this review. Medication use of participants with diabetes included statins [15,24], metformin, sulfonylureas [17] or insulin [24], while some were underwent dietary therapy alone [15,17,24]. Whether the use of medications affected the outcomes of interest is unclear, but is a potential confounding factor. As with all studies in populations with insulin resistance or diabetes, the variation in medications use treatments indicate that participants are likely to have different levels of glucose control and less likely to be comparable in terms of their physiological responses. The heterogeneity within and between these studies severely limits any conclusions surrounding the effect of a high AGE diet in patients with diabetes.

Though there were several studies which measured the effect of a high AGE diet on markers of inflammation and oxidative stress, and serum AGEs, due to differences in the way the results were reported and also missing n values for intervention groups in one study [25], it was not possible to perform a meta-analysis on the studies in this review.

Some studies reported use of vitamin supplements [15,24] while others did not [17,19,21–23,25] or asked participants to refrain from taking any supplements during the intervention period [16,18,20]. Vitamin B6 is a known AGE inhibitor [29] therefore failure to control for supplement intake could confound results. Several studies failed to report smoking status of the participants and did not list smoking as exclusion criteria [19,22,23,25]. This would represent a major confounder as tobacco smoke is another source of exogenous AGEs [44].

Dietary intake was poorly reported in several articles [15,21,22,25]. Some studies that relied on participants to prepare their own food reported significant differences in protein or fat intake between intervention groups [18,19,23]. Therefore in the studies that are missing dietary intake information and that did not provide food to participants [21,22,25] it is likely that there may have been differences other than the AGE content between interventions. Designing isocaloric diets that are matched for macronutrient and micronutrient content but contain varying AGE levels generated through cooking methods is challenging. However with thorough planning and controlled portion sizes confounding factors can be minimised [45]. Providing fully prepared meals to the participants adds significant strength to research into dietary AGEs however less than half of the studies reported here used this approach [15–17,20,24].

The majority of studies relied on a published AGE database [26] to determine the AGE content of the diet. However this database was generated using an enzyme linked immunosorbent assay (ELISA) that had not been validated against LC-MS and therefore may overestimate or underestimate the true AGE content of a food substance [46]. There is a database [10] of the AGE content of foods measured by LC-MS now available, although only a limited number of foodstuffs have been analyzed. Future research in this area should use databases which have AGEs measured by validated techniques.

Finally, the included studies had relatively short follow up duration (two to 16-weeks). The adverse consequences of a high AGE diet may arise over a period of years rather than weeks or months as AGEs accumulate, glycate other proteins, or are incorporated into tissues. Therefore, studies of longer duration are required.

4.3. Limitations of this Review

The risk of bias assessment tool used in this review used may have resulted in higher risk of bias or poorer quality ranking being assigned to studies that were more transparent with reporting. Also, limitation of the included studies to isocaloric diets resulted in the exclusion of one relevant and well reported trial [41]. Finally, the outcomes assessed here are biomarkers and do not necessarily predict end points or disease outcomes. Longer-term trials that include disease outcome are required before the effect of a high AGE diet on incidence of chronic disease can be confirmed. Also, a major concern highlighted in this review is that eight [15,19,21–25] of the included studies were performed by the same research group and therefore the external validity of the results of these studies is questionable. This then limits the generalisabilty of this review.

4.4. Comparison with Other Reviews

The conclusions drawn about the lack of high quality long-term trials in this field agree with previously published systematic reviews [2,3] despite limiting included studies to randomised controlled trials. The three most recent interventions [18,20,21] published after the earlier reviews were of variable quality. One of these recent studies [20] which was found to have a low risk of bias, addressed many of the issues highlighted by Kellow *et al.* [2]. Another, however, had major methodological flaws and confounding factors which resulted in a high risk of bias rating [18]. The overall findings that a high AGE diet may increase circulating TNFα, oxidative stress and circulating AGEs corroborate those reported in earlier systematic reviews and support the need for future high quality research in this area.

5. Conclusions

The findings of this review suggest that consumption of a high AGE diet increases circulating levels of TNFα and AGEs in healthy individuals and in individuals with chronic disease. Furthermore, there is evidence to suggest that dietary AGEs promote oxidative stress in healthy adults, and increase CVD markers in patients with diabetes. As such, dietary AGEs may play a role in the promotion of chronic conditions such as T2DM, CVD and CKD through increasing oxidative stress and inflammation.

The limitations of the current evidence, highlighted in this review, indicate that further high quality randomised controlled trials are required to fully delineate the adverse consequences of a high AGE diet in both healthy people and in patients with diabetes and CKD before any dietary recommendations can be made. Future studies into the effect of a high AGE diet, or benefit of a low AGE diet need to: (i) control for confounding factors (such as fat content or heat sensitive vitamins) between study diets; (ii) use validated methods to assess the AGE content of foods in the diet; (iii) provide participants with meals to increase compliance; (iv) include justifications of sample size; (v) include biomarkers of kidney function as primary outcomes; (vi) include methods to assess the metabolism of dietary AGEs in order to better elucidate the mechanisms by which a high AGE diet may have an adverse effect. Given that the standard Western diet contains a high amount of AGEs, further research into the consequences of habitual high AGE diets is important. The potential benefits of restricted AGE intake are promising and could offer a simple dietary therapy in the prevention and treatment of chronic conditions.

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Review

Selenium and Metabolic Disorders: An Emphasis on Type 2 Diabetes Risk

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Abstract: Selenium (Se) is a micronutrient that maintains biological functions through the action of Se containing proteins known as selenoproteins. Due to the known antioxidant effects of Se, supplements containing Se have been on the rise. While Se supplementation may be beneficial for Se deficient populations, few are at risk for Se deficiency due to the transportation of food from Se-rich regions and the rise of Se-enriched foods. Alarmingly, Se supplementation may have adverse effects in people who already receive an adequate Se supply. Specifically, an increased risk of type 2 diabetes has been reported in individuals with high baseline Se levels. However, this effect was restricted to males, suggesting the relationship between Se and glucose homeostasis may be sexually dimorphic. This review will discuss the current understanding of the interaction between Se and glucose homeostasis, including any sex differences that have been described.

Keywords: selenium; selenoproteins; metabolic disease; trace element

1. Introduction

Dietary Selenium (Se) is critical for the synthesis of selenoproteins, which carry out the biological functions of Se. To date, 24 murine and 25 human selenoprotein genes have been identified [1]. Of these gene products, the glutathione peroxidases and thioredoxin reductases, which participate in redox reactions, are likely the most well-studied selenoprotein families. Other notable selenoproteins with known functions include the iodothyronine family, which regulate thyroid hormone activation and Selenoprotein P (Sepp1), which is necessary for Se transport through the serum. Thus, selenoproteins function in a wide variety of processes (Table 1). Sources rich in Se include seafood, organ meats, dairy, grain, cereals, and Brazil nuts, albeit Se concentrations in plants are dependent on the Se levels in the soil, and the plant's capacity to uptake Se. Thus, populations that live in areas with low soil Se are at risk for Se deficiency. Complications from Se deficiency include Keshan disease [2], a cardiomyopathy, and Kashin-Beck disease [3], an osteocondrapathy. Low Se levels have also been implicated in male infertility [4]. The current daily recommended value for adults is 55 μg/day in the United States, a value which was determined based on maximal glutathione peroxidase activity [5]. The dose range for Se that is beneficial for human health is fairly narrow, and has been described as a U-shaped curve.

Se was long touted for its cytoprotective properties, due to its ability to upregulate antioxidant selenoenzymes. Thus, it was believed that Se supplementation could prevent the onset of metabolic diseases, such as type 2 diabetes (T2D), by counteracting oxidative stress. Indeed, Se in the form of selenate, was found to act as an insulin mimetic, displaying anti-diabetic effects [6]. In support of this, two cross-sectional studies reported lower baseline Se levels to be associated with T2D incidence among elderly French men [7], as well as in samples taken from a population in southeastern Spain [8]. A more recent, longitudinal study conducted in the United States, reported higher toenail Se to be associated with lower T2D risk [9]. However, other cross-sectional studies, namely the National Health and Nutrition Examination Survey (NHANES) III [10] and NHANES 2003–2004 [11], revealed an

association between high Se intake and metabolic disease. Moreover, increased T2D risk was found to be a secondary outcome in the Nutritional Prevention of Cancer (NPC) trial, a randomized, controlled trial assessing the efficacy of Se supplementation in the form of Se yeast (200 μg/day) in preventing skin cancer [12]. The Selenium and Vitamin E Cancer Prevention Trial (SELECT), testing the effects of selenomethionine (SMet, 200 μg/day) and/or Vitamin E in preventing prostate cancer was curtailed as it became apparent Se supplementation did not appear beneficial in the prevention of prostate cancer, and a nonsignificant trend towards T2D in the experimental group was reported [13,14]. Yet, other epidemiological studies and clinical trials failed to find a correlation between increased Se and T2D susceptibility [15]. One reason for the discrepancies in the human trials may be due to differences in baseline Se levels. For instance, the mean baseline Se levels in SELECT [13] subjects were already high (136 μg/L) whereas only the NPC [12] subjects in the upper third tertile of baseline Se (>122 μg/L) demonstrated higher incidence of T2D. Strengthening the idea of a narrow beneficial window of Se dose, it is likely that with regards to T2D, Se supplementation may be advantageous in populations with low Se status, but detrimental in Se-replete populations. In fact, randomized, controlled trials of Se supplementation in elderly patients [16] and pregnant women [17] from the UK, who have lower baseline Se than US subjects, did not result in increased T2D risk, as determined by serum adiponectin concentration. Another source of the inconsistencies might be attributable to differences in Se source. As discussed in detail below, different Se forms vary in their bioavailability and biological effects. Thus, it is difficult to delineate a clear-cut relationship between Se status and T2D based on evidence from the current human clinical trials and epidemiological studies.

Table 1. List of identified mammalian selenoprotein genes and their known functions.

Selenoprotein Gene	Abbreviation	Function(s)
15 kDa-selenoprotein	Sep15	Protein folding
Iodothrionine Deiodinase 1–3	Dio1–3	Thyroid hormone activity regulation
Glutathione Peroxidase 1–4, 6	GPx1–6	Hydroperoxide/phospholipid peroxide reduction
Methionine-R-Sulfoxide Reductase 1	MsrB1	Reduces oxidized methionine residues
Selenoprotein H	SelH	Genome maintenace
Selenoprotein I	SelI	Unknown
Selenoprotein K	SelK	ER-associated degradation; inflammation
Selenoprotein M	SelM	Ca^{2+} homeostasis
Selenoprotein N	SelN	Muscle development
Selenoprotein O	SelO	Unknown
Selenoprotein P	Sepp1	Selenium transport
Selenoprotein S	SelS	ER-associated degradation; inflammation
Selenophosphate Synthase 2	SPS2	Selenoprotein biosynthesis
Selenoprotein T	SelT	Ca^{2+} homeostasis; neuroendocrine secretion
Thioredoxin Reducase 1–3	TrxR1–3	Disulfide bond reduction
Selenoprotein V	SelV	Unknown
Selenoprotein W	SelW	Unknown

To obtain a clearer picture of the role of Se in metabolic disease, this review will focus on the current understanding of the connections between dietary Se, selenoproteins, and energy metabolism, with an emphasis on animal models. Particularly interesting is that the relationship between Se metabolism and glucose homeostasis appears to be sexually dimorphic, a topic that will also be discussed later in this review.

2. Forms of Selenium

Selenium occurs ubiquitously in the environment, but its biological activity is determined by the form that reaches an organism and how this form is metabolized. In general, the Se content of plants reflects the Se content of the surrounding soil and its bioavailability, and crop and livestock Se content will also reflect the Se content of the soil and the forage items they ingest [18]. Besides the dependence on the soil content, plant Se content may also vary according to pH and the presence of

soil ions that can form complexes with Se, enhancing or decreasing its bioavailability, according to the bacterial species present in the roots, and according to the ability of plants to uptake, accumulate and metabolize Se in its various forms [19–21].

During amino acid synthesis, plants generally employ Se and sulfur nonspecifically in their metabolic processes. Thus, most plants form methionine (Met) and SeMet in amounts that reflect the relative sulfur and Se concentrations of the soils in which they are grown [22]. The metabolic processes of SeMet and its downstream metabolites are generally analogous to those of Met in both plants and animals. Nevertheless, once incorporated in animal proteins in place of Met, the Se of SeMet can become part of an unregulated pool of Se, or be released when the amino acid is metabolized via methionine cycle or transsulfuration pathways, becoming part of the highly regulated selenocysteine (Sec) pool [23].

Efficient uptake and metabolism of dietary Se in animals will depend on which chemical form was ingested. Predominant forms of inorganic Se are selenite and selenate, both water-soluble [24]. Organic forms of Se mostly include the amino acids SeMet and Sec [25], and rare organic forms such as selenoneine, Se-methylselenocysteine, and selenoglutathione may have important biological roles that are currently unknown [20,26]. Inorganic selenite is absorbed by the enterocytes at rates that vary from 50% to 90% [23,27,28], depending on age, sex, and dietary constituents. However, the molecular mechanism responsible for Se absorption is poorly understood. Gastrointestinal selenate absorption is conducted transcellularly, possibly by the SLC26 multifunctional anion exchanger family, and paracellularly through the intestinal tract with virtually 100% efficiency [28–31]. Nevertheless, it is necessary for selenate to be reduced to selenite prior to being metabolized, and thus these inorganic forms possess lower retention rates when compared to organic forms [29,30,32]. In the case of organic SeMet, more than 90% is uptaken transcellularly by enterocytes [27,33]. Although the available data for Se absorption is limited, the European Safety Food Authority recently stated the Se absorption efficiency on a normal diet to be ~70% [34]. However, the United States National Institutes of Health guidelines does not state an overall absorption value for Se, due to the discrepancies in absorption rates of different Se forms [5].

Physiological to low-toxic doses of Se are known to be excreted as methylated sugar metabolites, specifically 1β-methylseleno-*N*-acetyl-D-galactosamine in the urine, while toxic levels of Se lead to the excretion of trimethylselenonium ion and dimethylselenide in the urine and breath [35].

The metabolic fates of organic and inorganic forms point to their conversion intracellularly to selenide to be utilized in selenoprotein synthesis [27,36]. Neither Sec nor SeMet accumulate in biological systems, Sec being promptly converted into selenide via decomposition of Sec by the enzyme Sec β-lyase (Scly), and SeMet via demethylation to methylselenol via γ-lyase activity [23,37,38].

Thio- and seleno-amino acids are structurally and metabolically analogous, differing in having either sulfur or selenium bound to the γ-carbon of their side chains. Nevertheless, they differ in physiological abundance, reactivity, acid dissociation constant (pKa) and metabolic roles. Met and SeMet are incorporated into proteins and peptides nonspecifically in amounts that reflect their tissue abundance. SeMet is broken down into methylselenol by the tetrameric enzyme cystathione γ-lyase (Cth), a member of the reverse transsulfuration pathway [39,40]. This pathway converts homocysteine to cysteine (Cys), which contributes to the generation of sulfide [41]. The methylselenol generated in the Cth-catalyzed reaction of SeMet can be demethylated and utilized in selenoprotein biosynthesis, including for the synthesis of GPx1 [42]. Because SeMet can contribute to the Se pool for selenoprotein biosynthesis, it is considered an additional source of biologically essential Se. Sec and Cys have analogous molecular structures, but Sec is exclusively inserted into genetically unique selenoprotein families, requiring a recoding of the UGA stop codon that will be further explored below. Interestingly, it has been uncovered that Cys can be incorporated in place of Sec in the enzymes thioredoxin reductase 1 (TrxR1) and 3 (TrxR3), using the Sec synthesis machinery. Cys incorporation encoded by UGA is enhanced in conditions of low dietary Se, suggesting a mechanism to reduce selenoprotein activity [43].

3. Selenoprotein Synthesis

Selenoprotein synthesis involves several processes that are distinct from normal protein translation. Eukaryotic Sec biosynthesis is a unique process beginning with the synthesis of Sec directly on its tRNA (Sec-tRNA[Ser]Sec) [44], requiring the aminoacylation of tRNA[Ser]Sec with serine via the action of seryl-tRNA synthetase. Phosphoseryl-tRNA kinase (PSTK) phosphorylates the resulting seryl-tRNA[Sec]Ser to form phosphoseryl-tRNA[Ser]Sec [45]. In a separate reaction, selenide is activated by selenophosphate synthetase 2 (SPS2), yielding selenophosphate [46,47]. The final step involves selenophosphate transfer onto phosphoseryl-tRNA[Ser]Sec by selenocysteine synthase (SecS), generating Sec-tRNA[Ser]Sec [48].

Perhaps one the most intriguing aspects of selenoprotein biosynthesis is the ability of Sec-tRNA[Sec]Ser to recognize the UGA codon, allowing for the recoding of what was conventionally thought to serve solely as a stop codon into a Sec insertion site. Consequently, intricate mechanisms have evolved to prevent premature translation termination. Sec insertion is dependent on the presence of a secondary structure known as the Sec Insertion Sequence (SECIS) element in the 3′ untranslated region of the selenoprotein mRNA [49]. The SECIS interacts with SECIS binding protein 2 (SBP2) [50,51], allowing for the recruitment of Sec-tRNA[Ser]Sec and Sec-specific elongation factor (EFSec) to the ribosome [52,53]. Other trans-acting factors have also been identified, including ribosomal protein L30 [54,55], SECp43, and SecS [56,57].

The presence of Se regulates selenoprotein synthesis. It was recently demonstrated through ribosome profiling that Se influence over selenoprotein synthesis occurs mainly through changes in translational efficiency [58]. Reduced Sec incorporation causes the UGA codon to be read as a premature stop codon, activating the nonsense-mediated decay (NMD) pathway [59]. However, it is important to note that selenoproteins are not affected equally by this pathway. Housekeeping selenoproteins such as TrxR1 and glutathione peroxidase 4 (GPx4) are more resistant to changes in Se levels, whereas the stress-response selenoproteins are more reactive to fluctuating Se status.

Sec decomposition occurs through Scly, a non-selenoenzyme that was initially purified from pig liver [60]. Scly has the ability to specifically distinguish Sec from the structurally similar amino acid, Cys [61]. In the presence of cofactor pyridoxal 5-phosphate, Scly cleaves Sec to form alanine and selenide. It was proposed that the selenide produced via Sec decomposition can be re-purposed for selenoprotein synthesis, implicating a role for Scly as a Se recycling enzyme when Se supply is low. In support of this, Scly was found to be required for selenoprotein biosynthesis *in vitro* when Sec is acting as the Se source [62]. Sec can be produced from selenoprotein degradation or alternatively generated by the transsulfuration pathway from selenomethionine [38]. However, Scly depletion did not hinder selenoprotein biosynthesis *in vitro* when selenomethionine was the sole source of Se [62], suggesting selenomethionine is able to contribute to the Sec pool through an alternate pathway. Moreover, Sec was found to donate Se to SPS to form selenophosphate, leading to the hypothesis that Scly and SPS enzymes work in a complex. *In vitro* immunoprecipitation studies confirmed Scly interaction with SPS1 and 2 [63]. The Se transport protein Sepp1, which contains multiple Sec residues, is synthesized primarily in the liver and secreted into the serum where it can be delivered to various tissues depending on need. Mice lacking both Scly and Sepp1 develop severe neurological deficits, surpassing the neurological phenotypes observed when only one gene is absent [64]. Hence, it has been suggested that Scly and Sepp1 act in tandem, with Sepp1 delivering Sec to tissues where Scly can cleave and recycle Se. Overall, the Sec decomposition pathway may also serve as a Se recycling pathway.

4. Selenium and Metabolic Disease

Although the association between Se supplementation and T2D in humans is considered to be controversial, studies in animal models may provide insights into some of the inconsistencies reported in human clinical trials. In a study comparing three different concentrations of Se in the diet, it was found that mice on a 0.4 ppm selenite diet developed insulin resistance, a hallmark of T2D [65]. This concentration of Se is comparable to the 200 μg Se regimen that was administered

to humans in the NPC trial. High Se exposure also led to insulin resistance in rats, which was attributed to both excessive ROS production and attenuated ROS [66]. Moreover, 16 weeks of Se supplementation in pigs on an already Se adequate diet resulted in a trend towards increased body weight and HOMA-IR score, a measure of insulin resistance [67]. This was accompanied by alterations in glucose and lipid metabolic pathways in insulin-sensitive tissues. With the exception of the skeletal muscle, glutathione peroxidase activity and thioredoxin reductase activities were largely unchanged in insulin-sensitive tissues in response to Se supplementation, suggesting selenoprotein expression was already saturated under Se adequate conditions. These results suggest the proclivity towards metabolic disease in pigs receiving supranutritional Se doses may be a result of nonspecific incorporation of SeMet, rather than a consequence of increased selenoprotein activity. It is important to note that most of the parameters measured in this study only trended towards significance, and thus it was concluded that supranutritional Se contributes to but does not cause T2D. However, as the duration of the study was relatively short, it is premature to speculate whether significance would have been achieved under long-term supplementation.

In vitro studies in pancreatic islets and the mouse insulinoma derived β-cell line, MIN6, have demonstrated increased insulin content and secretion in response to Se treatment, leading to the hypothesis that Se is protective in the endocrine pancreas [68]. Higher levels of plasma Se were indeed found to be associated with elevated serum insulin in mice [65], but it is not known whether this is protective or detrimental. However, selenate treatment conferred protection to rat insulinoma cells, INS1, against streptozotocin-induced β-cell death [69], suggesting the antioxidant properties of Se may be protective in the late stages of T2D.

The link between inflammation and T2D is well-documented. Briefly, pro-inflammatory cytokines, including Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)α, are elevated in T2D subjects, where they have been shown to promote insulin resistance in the adipose tissue, liver, and muscle, and β-cell failure in the pancreas [70]. The expression of these pro-inflammatory cytokines is dependent on the nuclear translocation of the transcription factor, nuclear factor-kappa B (NF-κB). As mentioned above, Se reportedly plays a physiological role in inflammation [71]. Notably, Se was found to inhibit the NF-κB pathway [72,73]. It is therefore conceivable that inflammation may link Se to T2D. In fact, selenite was able to rescue blood glucose levels of streptozotocin-induced diabetic rats [74]. In selenite-treated diabetic rats, hepatic NF-κB expression was found to be reduced, likely contributing to the improved phenotype. However, the necessity of the reduction in hepatic NF-κB expression in selenite-mediated reversal of streptozotocin effects were not tested. In a separate study, pharmacological doses of selenite administered to streptozotocin-induced diabetic mice partially rescued blood glucose levels [75]. Selenite-treated mice exhibited lower levels of pancreatic pro-inflammatory cytokines (IL-1β, TNF-α, and interferon (INF)-γ) and lipid peroxidation.

These studies demonstrate that both oversupplementation and deficiency of Se can be associated with T2D risk, following a U-shaped curve. It appears that Se oversupplementation may promote T2D in an otherwise healthy animal. However, in diabetic animals which may have suboptimal Se status, Se appears to have beneficial effects by preventing further development of T2D complications. Additional human studies will be useful in determining the appropriateness of Se supplementation with respect to T2D.

5. Selenoproteins in Metabolic Disease

Selenoproteins are important metabolites of dietary Se, fulfilling the catalytic effects of Se. Thus, in order to clarify the discrepancies in the association between Se supplementation and metabolic disease, it is necessary to understand the role of each selenoprotein in maintaining glucose homeostasis. This section will review what is currently known about the roles of selenoproteins that have been connected to metabolic disease.

5.1. Glutathione Peroxidase 1

Glutathione Peroxidase 1 (GPx1) is the primary cytosolic peroxide scavenger, reducing peroxides to water, and protecting the cell from free radical damage. Considered to be a stress responsive selenoprotein, GPx1 expression is sensitive to Se intake. Overexpression of GPx1 in mice yielded surprising results, leading to reduced glucose clearance, hyperinsulinemia, hyperglycemia, and diminished insulin signaling [76]. Diet restriction alleviated all metabolic symptoms except hyperinsulinemia, suggesting the functional role of GPx1 lies in regulating insulin production [77]. Indeed, it was found that the H3 and H4 histones in the proximal promoter region of the insulin gene transcription factor, pancreatic duodenal homeobox-1 (PDX1), were hyperacetylated, ultimately leading to hyperinsulinemia. Although not shown directly, the hyperacetylation was speculated to be due to enhanced H_2O_2 scavenging. The importance of H_2O_2 in cellular signaling was established further when it was demonstrated that GPx1 null mice have increased insulin sensitivity in the muscle, resulting in a high fat diet-resistant phenotype [78]. The absence of GPx1 allowed for the oxidation of phosphatase and tensin homolog (PTEN), a member of the PTP family. Oxidation of PTEN inhibits its activity, sensitizing insulin signaling.

In contrast, several studies suggest that GPx1 might play a protective role against T2D. GPx1 overexpression in HIT-T15 cells protects against β-cell dysfunction induced by ribose treatment [79]. As ribose was found to induce oxidative stress in human islets, it is possible that GPx1 promotes β-cell survival through its ability to scavenge peroxides. Although constitutive GPx1-/- mice appear to preserve insulin signaling in response to an obesogenic diet, in the context of insulin secretion, GPx1 deficiency can be detrimental [80]. In the absence of GPx1, excess ROS in the pancreatic islets oxidize PTPN2, promoting STAT1 signaling which results in downregulating key enzymes of the insulin production and secretory pathway, such as Pdx1. Inactivation of hepatic PTPs due to excess ROS also appears to promote obesity and T2D disease progression. In hepatocytes isolated from GPx1-/- mice, the presence of excess ROS inactivates PTPN2, which negatively regulates STAT5-induced lipid synthesis [81]. Thus, hepatic GPx1 may prevent hepatic steatosis indirectly by regulating ROS levels. Further supporting the protective function of GPx1, the GPx mimetic, ebselen, was found to restore islet function in GPx1-/- mice through a PGC-1α dependent mechanism [82]. Nrf2 is a transcription factor that controls the transcription of antioxidant enzymes by interacting with an antioxidant response element (ARE). Several selenoproteins and selenoprotein synthesis factors were found to contain an ARE in their promoters, and are thus Nrf2-responsive. GPx1 levels were found to be suppressed when mice were fed a high fat diet due to 12-lipoxygenase activity, which was found to inhibit Nrf2 nuclear translocation [83]. Pancreatic islet specific 12-lipoxygenase deletion resulted in GPx1 upregulation in response to high fat feeding. The resulting reduction in oxidative stress was found to be beneficial in preserving islet β-cell function under high fat diet consumption in mice. To corroborate these studies, a genetic polymorphism in the GPx1 gene which results in lower GPx activity, was found to correlate with increased incidence of metabolic syndrome in a cohort of Japanese men [84].

Generally speaking, both GPx1 overexpression and deficiency appear to have negative effects in metabolic disease. These findings are aligned with the U-shaped therapeutic dose effect of Se intake. However, the function of GPx1 is tissue dependent, exhibited by the differences in outcome of GPx1 deficiency in the muscle [78], liver [81], and pancreatic islets [80]. Increased understanding of tissue-specific functions of GPx1 will improve our knowledge of the role GPx1 plays in metabolic disease. It is also important to note the GPx1 response may also have a timing specific component in that excess GPx1 in the pre-disease state may promote disease pathogenesis. The disease state, however, may suppress GPx1 expression, thus, targeting GPx1 pathways may be beneficial.

5.2. Selenoprotein P

In humans, a positive correlation between hepatic SEPP1 expression and T2D has been reported [85]. In this same study, increased hepatic SEPP1 mRNA expression was also associated with reduced glucose tolerance and higher fasting glucose levels, which are indicative of insulin resistance.

However, it is important to note that serum Sepp1 levels become saturated at high Se intake [86]. One limitation of this study [85] is that the patients' Se intake levels were not reported. Likely, the usefulness of Sepp1 as a biomarker is limited to subjects who do not receive an optimal Se intake. Additionally, since Se deficiency has been reported in T2D patients [7], it is possible that Sepp1 mRNA expression is elevated in the diseased state, as Se transport will be in higher demand. Thus, the elevation of Sepp1 may be a secondary effect of T2D, rather than a cause. Nevertheless, adiponectin, an adipokine with anti-diabetic effects [87], was found to be inversely correlated with serum Sepp1 levels in T2D patients [88]. Moreover, elevated serum Sepp1 levels were positively correlated with carotid intima-media thickness and C-reactive protein, both of which are predictors for cardiometabolic disease [89]. Studies of SEPP1 genetic variants reveal SEPP1 polymorphisms to be associated with fasting insulin and the acute insulin response [90]. Taken together, Sepp1 appears to be involved in glucose homeostasis, although direct conclusions cannot be made due to the correlative nature of these studies. Mouse models and cell lines have been used to delineate the mechanistic relationship between Sepp1 and carbohydrate metabolism. For example, studies in HepG2 cells revealed that hepatic Sepp1 mRNA and promoter activity are under the control of insulin and a supramolecular complex composed of PGC1α, FoxO1a, and HNF-4α, which also regulates gluconeogenic enzymes PEPCK and G6Pase [91]. Additionally, Sepp1 was found to negatively regulate insulin signaling in the liver, through AMPK inactivation in female mice [85]. Sepp1 also downregulates insulin signaling in the muscle, although the mechanism remains unclear. Mice with Sepp1 deletion (Sepp1-/-) were found to be protected from diet induced obesity and insulin resistance. In a follow-up study, Sepp1-/- mice were found to be protected from the drop in serum adiponectin levels in response to a high-sucrose, high-fat diet, although adiponectin levels were not completely restored to wild-type. This implicates a partial, but direct role for Sepp1 in regulating adiponectin [88]. Because Sepp1 is associated with gluconeogenic enzymes and downregulation of the insulin signaling pathway, it was proposed as a potential drug target. In fact, the commonly prescribed glucose lowering drug, metformin, suppresses Sepp1 expression [92]. Further investigation demonstrated that metformin-induced inhibition of Sepp1 expression occurs in an AMPK and FoxO3 dependent pathway [93].

5.3. Selenoprotein M

Selenoprotein M (SelM) is localized to the endoplasmic reticulum (ER) and is thought to participate in thiol-disulfide exchange through its thioredoxin-like domain [94]. *In vitro*, SelM has been shown to regulate calcium signaling and protect against oxidative stress [95]. Because of its high expression levels in the brain, it was initially hypothesized that SelM offered neuroprotective properties. However, no deficits in learning and memory were observed in SelM knockout (SelM-/-) mice under a Se adequate diet [96]. Interestingly, SelM deletion in mice results in adult-onset body weight gain and increased adiposity, suggesting SelM may play a role in obesity. Immunohistochemistry further supported this hypothesis, as SelM was revealed to be highly expressed in the paraventricular nucleus and the arcuate nucleus of the hypothalamus, regions that are implicated in energy homeostasis. The arcuate nucleus contains neurons expressing the leptin receptor, which is activated by the adipocyte-derived peptide, leptin [97]. The downstream effects of the leptin receptor are carried out via the Jak2-Stat3 pathway. Leptin resistance in the hypothalamus leads to metabolic disease. Although a direct mechanistic relationship remains to be tested, SelM may play a role in energy metabolism through regulating leptin signaling. Whole body SelM deletion in mice results in elevated circulating leptin levels and diminished phosphorylated Stat3 levels in the hypothalamus, which are indicative of leptin resistance [96]. Furthermore, ER stress has been implicated in hypothalamic leptin resistance [97]. As SelM is an ER-resident protein, there is a possibility SelM may promote leptin signaling by protecting against ER stress. Currently, it is unknown whether SelM contributes to human obesity. Given the possibility that SelM may promote leptin signaling by mitigating ER stress, further investigation into this relationship is warranted.

5.4. Iodothryonine Deiodinase 2

Low Se levels have been associated with thyroid disorders such as goiter [98,99]. Moreover, thyroid hormones exert strong effects on obesity. The primary thyroid hormone in the bloodstream is L-3, 3′, 5, 5′ tetraiodothyronine or thyroxine (T4), a pro-hormone with four iodines and a long half-life. To become biologically active, one iodine is removed from T4, producing L-3, 5, 3′ triiodothyronine (T3). Thyroid hormone deiodination is catalyzed by a selenoprotein family, the iodothyronine deiodinases (Dio). Dio1 and Dio2 mostly convert T4 into active T3, and Dio3 converts T4 into inactive reverse T3 (rT3), or T3 into T2, leading to either inactivation or degradation of thyroid hormone. Local deiodination via Dio enzymes allow tight, controlled regulation of thyroid hormone levels [100].

Thyroid hormones are known for the regulation of metabolism and basal metabolic rate via regulation of energy expenditure, thus tight control of thyroid hormone levels can dictate effects on energy balance [101,102]. Diet-induced obesity in male mice has also been demonstrated to depend on Dio2 activity in the anterior pituitary activated by the c-Jun N-terminal kinase (JNK) pathway, controlling TSH levels and consequent thyroid hormone-dependent energy expenditure [103]. Moreover, mice with targeted deletion of Dio2 in the pituitary have less body fat, despite maintaining their oxygen consumption normally [104].

Specifically, Dio2 controls adaptive thermogenesis induced by cold and by diet in the brown adipose tissue. Mice with targeted disruption of Dio2 (Dio2 KO) lack proper adaptive thermoregulation [105–107] and are more prone to high fat diet-induced obesity [108]. Male and female Dio2 KO mice are insulin resistant even on a normal chow diet, with increased gluconeogenesis, and accumulate triglycerides in the liver, despite not yet displaying significant weight gain. Dio2 activity also controls feeding at the arcuate nucleus of the hypothalamus via local control of T3 levels, which contributes further to energy balance [109,110]. Inability to properly activate thyroid hormone via Dio2 was linked to glucose intolerance through hepatic insulin resistance. Intriguingly, human Dio2 gene expression is inhibited by the heterodimerization of liver X-receptor (LXR) with the retinoid X-receptor (RXR) [111]. Dio2 regulation by a classic lipogenic transcription factor was observed in LXRα and LXRβ double KO mice, which ectopically express Dio2 in the liver [112], suggesting a role for Dio2 inhibition in hepatic lipid deposition and obesity.

5.5. Selenoprotein T

Selenoprotein T (SelT) was first identified *in silico*, using an algorithm to identify SECIS elements in the human dbEST [113]. Containing a thioredoxin-like fold, SelT was proposed to possess redox activity [114], but its precise function remains unknown. Bioinformatics analysis revealed SelT to be localized to the ER, possibly being trafficked to the plasma membrane [115]. SelT expression in mice appears to be highest during development, with SelT mRNA expression in most tissues decreasing in adulthood, except in endocrine tissues such as the thyroid, pituitary, testis, and thymus [116]. The pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide which increases cAMP through adenylate cyclase stimulation, having implications in a variety of cellular processes, including cell survival and secretory function. SelT was recently identified as a target of PACAP [117]. In differentiated PC-12 cells, SelT is necessary for PACAP-dependent neuroendocrine secretion by regulating intracellular Ca^{2+} levels. Many neuropeptides regulate energy homeostasis, such as NPY (neuropeptide Y) [118], Agrp (agouti-related peptide) [119], α-MSH (α-melanocyte stimulating hormone) [120], among others. Identifying the neuropeptides under SelT regulation could provide greater understanding of the connection between dietary Se and energy metabolism. Immunofluorescence demonstrated SelT to be highly expressed in the adult pancreatic β and δ-cells, the latter of which secretes somatostatin [121], indicating SelT may be involved in glucose homeostasis. Conditional knockout of SelT in the β-cell resulted in defective insulin secretion, suggesting SelT is critical to β-cell function. Studies in the glucose responsive murine β-cell line, MIN6, determined that PACAP-induced insulin secretion depends on SelT expression. This indicates that SelT may regulate

blood glucose at multiple levels. Although SelT is expressed in other metabolic tissues such as the pituitary and thyroid [116], the function of SelT in these tissues is unknown.

5.6. Selenoprotein S

Like SelT, Selenoprotein S (SelS) was first identified *in silico*, and was shown to localize to the plasma membrane [1]. Functionally, SelS has implications in ER-associated degradation (ERAD) [122], inflammation [123], and the transport of multi-protein complexes [124]. In 2003, a novel protein, Tanis, was characterized as a glucose-regulated protein in *Psammomys obesus*, an animal model for T2D [125]. Tanis was found to be expressed in insulin-sensitive tissues such as adipose tissue, liver, and skeletal muscle. Through yeast two-hybrid screening, Tanis was found to interact with serum amyloid A, a family of proteins associated with the acute-phase inflammatory response, which is typically elevated in T2D patients. Potentially, Tanis acts as a receptor for serum amyloid A. Tanis was later identified to be a SelS homolog [1], leading to the hypothesis that SelS links inflammation to T2D. In support of this, a positive correlation between serum amyloid A levels and SelS expression in the skeletal muscle and adipose tissue of T2D patients was reported [126]. SelS appears to be dysregulated in the disease state, as insulin stimulation increases SelS mRNA expression in the adipocytes of T2D subjects but not healthy subjects. Conversely, a different study found subcutaneous adipocyte SelS mRNA expression to increase in response to insulin in both obese and lean subjects [127]. This study also failed to find a correlation between serum amyloid A and SelS expression. However, SelS expression was found to be higher in obese subjects, with increased subcutaneous SelS expression in obese subjects associated with BMI, sagittal diameter, serum HDL, triglycerides, insulin, and insulin resistance. Additionally, SelS polymorphisms were correlated with higher diastolic blood pressure and circulating insulin. These individuals were also at a higher risk for cardiovascular disease. Taken together, these studies support the role of SelS in metabolic disease.

While SelS has been associated with T2D, its role in metabolic disease remains unknown. One possibility is that SelS plays a protective role. For instance, SelS has been shown to be upregulated in the hepatoma-derived HepG2 cells in response to glucose deprivation [128], albeit the physiological relevance is debated, as the low glucose concentration tested was 2 mM, well below the range of normal blood glucose levels in humans. However, SelS was also found to increase in response to ER stress, while overexpression of SelS conferred protection against oxidative stress MIN6 cells. Thus SelS may play a protective role, counteracting oxidative stress in T2D development.

6. Selenium Metabolism in Metabolic Disease

Generation of the Scly knockout (Scly-/-) mouse model established a connection between Sec decomposition and metabolic syndrome for the first time. The Scly-/- mice develop a metabolic syndrome-like phenotype under a Se adequate diet, displaying hyperinsulinemia, increased body weight, dyslipidemia, and reduced glucose tolerance [129]. Under these conditions, hepatic selenoprotein levels are unchanged, suggesting that Scly may function in glucose metabolism in a role that is independent from its ability to regulate selenoproteins. However, a mechanistic link between Scly and glucose metabolism has yet to be determined. The metabolic phenotype in the Scly-/- mice is exacerbated under Se deficient conditions [129], strengthening the hypothesis that Scly is involved in Se recycling during Se deficiency. Not surprisingly, likely due to the inability to recycle Se, Scly-/- mice have diminished hepatic expression of GPx1 and SelS, as well as diminished serum Sepp1. It is interesting to note that these selenoproteins have been implicated in metabolic disease. A more comprehensive analysis of the altered selenoprotein expression in Scly-/- mice may provide a better understanding of the selenoproteins that are influenced in metabolic syndrome.

High fat diet studies demonstrated that Scly-/- mice are more susceptible to diet-induced obesity than their wild-type counterparts [130]. Because the high fat diet given to the mice consisted of an adequate Se supply, it is not surprising that Scly-/- mice and wild-type mice on a high fat diet had similar hepatic selenoprotein profiles. Intriguingly, serum Sepp1 levels were elevated in Scly-/- mice,

possibly indicating increased gluconeogenesis. This strengthens the notion that Se and carbohydrate metabolic pathways are interconnected. Investigation of TCA cycle parameters revealed that Scly-/- mice fed a high fat diet have elevated levels of pyruvate, pyruvate dehydrogenase, the gluconeogenic enzyme, pyruvate carboxylase, and the lipogenesis promoting acetyl-CoA carboxylase. Additionally, citrate synthase activity was increased in Scly-/- mice. These findings suggest that in addition to carbohydrate metabolism, Se metabolism also regulates lipogenic pathways.

7. Sex Differences in Se and Metabolic Disease

Sex differences in Se uptake and selenoprotein expression patterns have been described and are reviewed elsewhere [131]. Given the sexual dimorphism in Se regulation, it is unsurprising that human clinical trials hint at the possibility that the relationship between Se and T2D is sex-specific. In the NPC trial, the increased T2D risk in response to Se supplementation was limited to males [12], although one limitation of this study is that females were severely underrepresented, comprising only 25% of the subjects. Nevertheless, the NHANES III, found a correlation between T2D risk and high Se in males, but not females [10], a finding which was supported by NHANES 2003–2004 [11]. In contrast, but still supporting the hypothesis that the Se and T2D interrelationship is sex-specific, Akbaraly *et al*. [7] reported a correlation between lower baseline Se and T2D incidence among elderly French men, but not women. Understanding sex differences in the biological function and regulation of selenoproteins may explain the sexually dimorphic results of Se and T2D. Unfortunately, studies involving sex differences in the contribution of individual selenoproteins to metabolic disease are limited. This section will highlight some of the known sex differences reported in selenoprotein regulation of energy metabolism.

GPx1 polymorphisms were correlated with increased MetS incidence in Japanese men, but not women [84]. The initial studies that investigated obesity and hyperinsulinemia in GPx1 overexpressing mice only utilized male mice, thus it is unknown whether the effect of GPx1 overexpression in mice is sex-specific [76,77]. Serum Sepp1 levels were elevated in diabetic men and women compared to healthy subjects [85]. However, subsequent studies investigating Sepp1 and insulin resistance involved female mice. Whether Sepp1 directly induces insulin resistance through AMPK in male mice is unknown. In the same study, Sepp1 deficiency was found to produce an obesity resistant phenotype in male mice. Female mice were left out due to inconsistencies in the results. Both male and female SelM-/- mice demonstrated an increase in body weight and adiposity when compared to wild-type mice [96]. However, the increase in circulating serum leptin was limited to male SelM-/- mice, suggesting that SelM regulation of leptin signaling is sex-specific. Moreover, since both male and female SelM-/- mice develop obesity, the implication is that the development of obesity in these mice occurs through sex-specific pathways. Although sex differences in the association between SelS and metabolic diseases have not yet been described, there are sex differences in the amount of Se necessary to reach maximal murine hepatic SelS expression [132]. This discrepancy in SelS expression may contribute to the differences observed in the effectiveness of Se supplementation in a model of septic shock. We must not exclude the possibility that the sex differences in the regulation of SelS expression might result in sex-specific outcomes in metabolic disease.

The studies in Scly-/- mice were conducted in males as it was observed that female Scly-/- tended to display a mild metabolic phenotype, whereas the differences in males were more pronounced [129]. A plausible explanation is that the Se metabolic pathway does not interfere with energy metabolism in females. Recent evidence from mice with combined Scly and Sepp1 deletion (Scly-/-Sepp1-/-) demonstrates that female mice are less dependent on the Se recycling pathway for neurological function, as female Scly-/-Sepp1-/- mice do not exhibit the neurological deficits reported in male Scly-/-Sepp1-/- mice [64]. As the primary Se source for the brain and testes, Sepp1 is particularly critical for these tissues, supplying Se via the ApoER2 receptor [133,134]. Absence of either Sepp1 or ApoER2 results in a similar phenotype, consisting of behavioral deficits and male infertility. Strikingly, castration of male Scly-/-Sepp1-/- mice was shown to attenuate the neurological dysfunction present

in uncastrated mice, offering a novel representation of the competition between the brain and testes for Se supply [135]. In the context of metabolic disease, it is conceivable that sequestration of available Se by the testes occurs at the expense of metabolic tissues, leading to altered glucose homeostasis in males.

8. Final Remarks

Se undoubtedly has numerous benefits to human health, with implications in T2D [15], cancer [136], male fertility [137], and neurological function [138], among others. However, the association of high Se intake and T2D in human epidemiological studies and clinical trials raises concerns regarding the practicality of Se supplements. Studies in animal models have been valuable in understanding that the effects of Se intake on T2D are dose-dependent, and perhaps plays contrasting roles in the diseased *versus* non-diseased state. Animal studies have also offered mechanistic insight, identifying potential links between Se and T2D, namely oxidative stress and inflammation. In addition, because the effects of Se occur through the enzymatic actions of selenoenzymes, the individual selenoproteins (GPx1, Sepp1, SelM, Dio2, SelT, SelS) that have been connected to T2D provide important information on the details of Se in T2D. Moreover, the Se metabolic pathway has been suggested to influence carbohydrate and lipid metabolism, strengthening the idea that Se does in fact play a role in T2D. However, the aforementioned sexually dimorphic association between Se and metabolic diseases reveals the complexity of Se processes. In order to reduce potentially undesirable effects of Se supplementation, and to further improve dietary guidelines for Se, improved understanding of Se metabolism and selenoproteins in both male and female subjects is essential.

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Abbreviations

The following abbreviations are used in this manuscript:

α-MSH	α-melanocyte stimulating hormone
Agrp	Agouti-related peptide
ARE	Antioxidant response element
Cth	Cystathione γ-lyase
Cys	Cysteine
Dio	Iodothyronine deiodinases
EFSec	Sec-specific elongation factor
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GPx1	Glutathione peroxidase 1
GPx4	Glutathione peroxidase 4
IL	Interleukin
JNK	c-Jun N-terminal kinase
LXR	Liver X-receptor
Met	Methionine
NF-κB	Nuclear factor-kappaB
NMD	nonsense-mediated decay
NPY	Neuropeptide Y
PACAP	Pituitary adenylate cyclase

PDX1	pancreatic duodenal homeobox-1
PSTK	Phosphoseryl-tRNA kinase
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
rT3	Reverse T3
RXR	Retinoid X-receptor
SBP2	SECIS binding protein 2
Scly	Selenocysteine lyase
Se	Selenium
Sec	Selenocysteine
Sec-tRNA[Ser]Sec	Sec tRNA
SECIS	Sec insertion sequence
SelM	Selenoprotein M
Sepp1	Selenoprotein P
SelS	Selenoprotein S
SelT	Selenoprotein T
SeMet	Selenomethionine
SPS2	Selenophosphate synthetase 2
T2D	Type 2 diabetes
T3	L-3, 5, 3′ triiodothyronine
T4	thyroxine or 5, 5′ tetraiodothyronine
TNF	Tumor necrosis factor
TrxR1	Thioredoxin reductase 1
TrxR3	Thioredoxin reductase 3

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Review

The Potential Protective Action of Vitamin D in Hepatic Insulin Resistance and Pancreatic Islet Dysfunction in Type 2 Diabetes Mellitus

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Abstract: Vitamin D deficiency (*i.e.*, hypovitaminosis D) is associated with increased insulin resistance, impaired insulin secretion, and poorly controlled glucose homeostasis, and thus is correlated with the risk of metabolic diseases, including type 2 diabetes mellitus (T2DM). The liver plays key roles in glucose and lipid metabolism, and its dysregulation leads to abnormalities in hepatic glucose output and triglyceride accumulation. Meanwhile, the pancreatic islets are constituted in large part by insulin-secreting β cells. Consequently, islet dysfunction, such as occurs in T2DM, produces hyperglycemia. In this review, we provide a critical appraisal of the modulatory actions of vitamin D in hepatic insulin sensitivity and islet insulin secretion, and we discuss the potential roles of a local vitamin D signaling in regulating hepatic and pancreatic islet functions. This information provides a scientific basis for establishing the benefits of the maintenance, or dietary manipulation, of adequate vitamin D status in the prevention and management of obesity-induced T2DM and non-alcoholic fatty liver disease.

Keywords: calcitriol; glucose homeostasis; HepG2; hypovitaminosis D; insulin secretion; lipid metabolism

1. Introduction

The liver is a vital organ for metabolic homeostasis, controlling glucose uptake-storage-generation and processing about one third of consumed glucose, and it is a key target for insulin action [1]. Insulin controls lipogenesis (fatty acid and triglycerides biosynthesis) and restrains hepatic gluconeogenesis (glucose production) in the liver. Accordingly, insulin sensitivity has been closely associated with rates of hepatic gluconeogenesis and lipid accumulation [2,3]. Hepatic insulin resistance leads to severely dysregulated glucose homeostasis, resulting in or contributing to hyperglycemia, which then further worsens hepatic insulin insensitivity. This negative cycle progresses toward a pathologic state of hepatic dysfunction and eventually liver disease. Notwithstanding these observations, it is unclear whether hepatic insulin resistance is a cause or consequence of type 2 diabetes mellitus (T2DM), and the underlying mechanism(s) mediating the relationship between hepatic insulin resistance and T2DM remain elusive. In addition to conventional regulatory factors (e.g., genes encoding glycolytic and lipogenic enzymes), changes in life style and nutritional factors have garnered ever increasing attention in the development of preventative and therapeutic approaches to both T2DM and non-alcoholic fatty liver disease (NAFLD). In this context, recent investigations into the interactions of nutritional factors, such as vitamin D, in T2DM-related diseases and their management are of particular interest in terms of both clinical and socio-economic impacts [4–6].

The endocrine pancreas (functionally distinct from the exocrine portions important for production of digestive enzymes) contains insulin-secreting pancreatic islets [7]. Dysfunction or loss of beta cells in pancreatic islets leads to hyperglycemia and hyperlipidemia, two predominant indicators of T2DM [8].

Islet physiology is finely modulated by a myriad of factors, including vitamin D. Vitamin D deficiency, referred to clinically as hypovitaminosis D, impairs insulin secretion and increases insulin resistance, key features of T2DM and related metabolic diseases [9,10]. Interestingly, glucose-stimulated insulin response experiments suggest that early vitamin D supplementation may be protective of insulin secretory function, whereas late supplementation, after vitamin D deficiency and T2DM have been established, may be relatively ineffective [11]. However, the precise modulatory roles of vitamin D in hepatic and islet functions have not been determined.

The purpose of the present review is to provide a critical appraisal of our current knowledge related to the protective effects of vitamin D on hepatic metabolism and islet function, particularly in the context of T2DM and obesity-associated diseases. Such information will be important for informing healthcare providers and patients about the potential benefits of vitamin D supplementation, as a food additive or nutraceutical. In particular, this work focuses on the potential of vitamin D supplementation to oppose the development or worsening of obesity-related diseases, such as T2DM and NAFLD, and thus provide a cost-effective measure for improving hepatic and pancreatic islet functions.

2. Vitamin D Synthesis and Metabolism

The pathophysiology of the insulin-resistant state and islet dysfunction remains enigmatic. Currently, patients with insulin resistance and islet dysfunction are treated with insulin sensitizers and secretagogues, which work mainly by improving glucose disposal via skeletal muscle and suppressing hepatic gluconeogenesis, and by stimulating pancreatic beta-cell insulin secretion, respectively [12]. Mechanism-based studies of the factors that influence insulin secretion and resistance, such as niacin [13,14] and vitamin D (*vide infra*), are needed to identify novel therapeutic targets.

Classically, vitamin D is known for its involvement in the regulation of calcium and bone homeostasis. There are two major forms of vitamin D, namely cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2). The primary source of vitamin D in humans is from endogenous biosynthesis in skin cells, a critical step of which hydroxylation of 7-dehydrocholesterol into cholecalciferol is catalyzed by ultraviolet radiation from sunlight [15]. Additionally, cholecalciferol can be found in dietary sources, including fish, egg yolk, beef, and lichen, while ergocalciferol can be found in edible mushrooms and alfalfa. Endogenous and dietary vitamin D molecules, which are biologically inactive themselves, are transported systemically in chylomicrons and converted into the metabolically active 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) via a sequence of reactions. In the liver, vitamin D is converted into 25-hydroxyvitamin D ($25(OH)D_3$), the clinically monitored form of plasma vitamin D, by the liver-derived enzyme 25-hydroxylase. Subsequently, $25(OH)D_3$, which is considered a prehormone, is then further hydroxylated to biologically active $1,25(OH)_2D_3$ by kidney-derived 1-α-hydroxylase [16].

The active form of vitamin D, $1,25(OH)_2D_3$, binds vitamin D receptors (VDRs) and activates various transcription factors. For example, activated VDR forms a heterodimer with retinoic X receptor (RXR), and thereby participates in stimulating the RXR nuclear pathway [17]. Indeed, classical vitamin D response elements and other response sites are broadly distributed and critical for pancreatic beta-cell and immune system functions [18–21]. Such findings suggest that manipulations of VDR signaling pathways might be physiologically relevant for the management of diabetes. Figure 1 is a summary of vitamin D synthesis and metabolism, as well as the modulatory action on islet function.

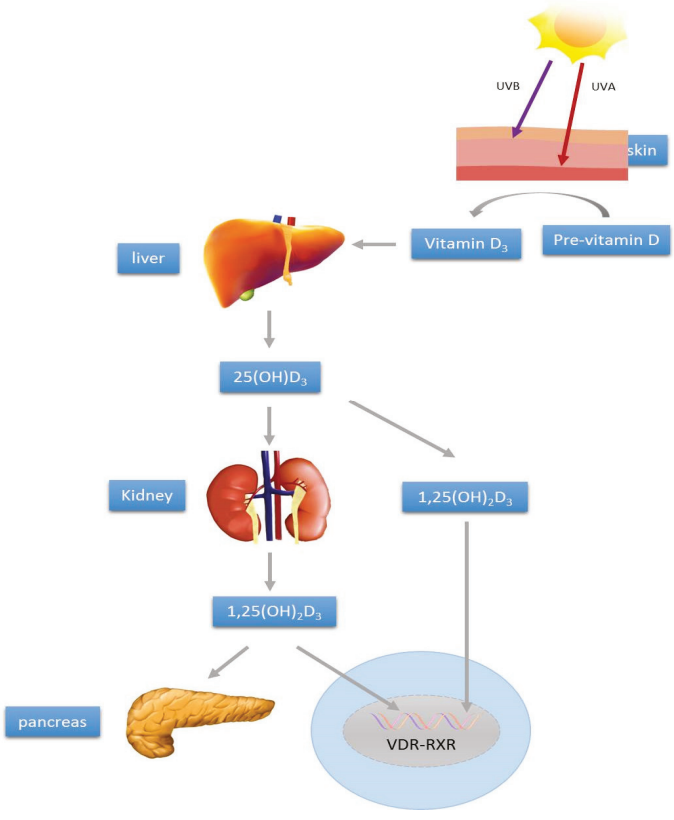

Figure 1. Schematic representation of vitamin D synthesis and metabolism in relation to regulation of pancreatic islet function and survival.

3. Vitamin D and T2DM

Hypovitaminosis D has been correlated with increased risks of a wide variety of chronic diseases. The major causes of hypovitaminosis D are sunlight deprivation (such as by sunscreen, melanin, latitude, and winter), medications and supplements, and malabsorption (such as in Crohn's and celiac diseases, cystic fibrosis, and liver disease). Maintenance of plasma $25(OH)D_3$ above 30 ng/mL is recommended for optimal health; some may require dietary supplementation and/or increased exposure to sunlight to attain this level [15]. Of great interest for our present focus, hypovitaminosis D has been reported to impair islet insulin secretion and to increase peripheral insulin resistance, two major risk factors for progression to T2DM; furthermore, hypovitaminosis D is predictive of abnormalities in most of the variables monitored in patients with metabolic syndromes, including T2DM itself [22,23]. It is worthwhile emphasizing that the clinical association between hypovitaminosis and insulin resistance/T2DM might not be a cause-and-effect relationship and that an increase in circulating $25(OH)D_3$ concentration might not necessarily reduce the onset of, or progression to, T2DM. In this context, there are considerable randomized clinical trials showing disassociation between plasma levels of active $25(OH)D_3$ and the incidence of T2DM, as recently exemplified by a mendelian randomization-derived estimates for glycemic control [24]. In fact, maintenance of adequate vitamin D status and/or its high-dose supplementation have not convincingly displayed long-term glycemic control for human T2DM in several clinical trial studies [25–28]. Such negative studies or discrepancies

have yet to be validated by further large-scale clinical studies with large sample sizes, different study designs (cross-sectional and interventional studies, and different populations), as well as by intensive mechanism-driven basic science studies in the future.

T2DM, which is defined as hyperglycemia of sufficient magnitude to cause detrimental effects, results when insulin resistance develops and is followed by dysregulation of insulin secretory responses, with loss of beta-cell mass [29]. Amelioration of both insulin resistance and islet dysfunction are, therefore, both of paramount importance for preventing and treating T2DM. Chronic hyperglycemia and hyperlipidemia are associated with dramatic upregulation of lipid formation and accumulation in the liver and pancreatic islets. Excessive hepatic lipid accumulation causes endoplasmic reticulum (ER) stress and inflammation (lipotoxicity), as well as reduced insulin sensitivity [30,31]. Meanwhile, an increase in intra-islet lipid accumulation impairs glucose-stimulated insulin secretion, and promotes islet inflammation and ER stress, ultimately leading to beta-cell apoptosis and islet failure [32].

Given the importance of vitamin D status for the maintenance of a physiologically healthy liver and pancreas, together with the known involvement of hepatic and pancreatic pathophysiology in T2DM pathogenesis, vitamin D and the VDR represent an area of great therapeutic interest. Identification of agents that can reduce abnormalities of hepatic and islet metabolism simultaneously could yield a substantial advancement in the prevention and treatment of obesity and obesity-related T2DM risk. To this end, there has been a surge of interest in the benefits of maintaining an adequate vitamin D status and the apparent protective mechanism(s) of vitamin D.

4. Vitamin D and Hepatic Metabolism

The liver is a vital organ consisting of functional units called liver lobules, which in turn are composed principally of hepatic cellular plates. The major cell types of the liver are hepatocytes, stellate cells, Kupffer cells, and endothelial cells, with hepatocytes being the functionally predominant cells [1]. As mentioned above, the liver is a major target organ of insulin, wherein insulin regulation helps to maintain glucose homeostasis by making glucose available when it is needed through gluconeogenesis and glycogenolysis, and by storing glucose through glycogenesis when it is present in excess [1]. Gluconeogenesis rate is determined primarily by the transcriptional level of the genes encoding two gluconeogenic enzymes, namely phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PK) [33]. The opposing process of glycogenesis is mediated by glycogen synthase (GS); GS activity is promoted when glycogen synthase kinase 3 (GSK-3) activity is inhibited [34].

Glucose and lipid metabolism are critical inter-related components of glucose homeostasis. When glucose intake exceeds storage and oxidation capacities, it is converted to fat (*de novo* lipogenesis); however, excessive hepatic lipid causes inflammation and hepatic insulin resistance [35]. Interestingly, numerous studies have suggested that ER stress, which is caused by an imbalance between protein folding stress and the processing capacity of the ER, is closely associated with metabolic disorders. In particular, hepatic ER stress promotes hepatic glucose production, lipogenesis, and insulin resistance in obese and diabetic states [36]. Hence, maintenance of normal hepatic cellular metabolic function appears to be indispensable for preventing the development of hepatic insulin resistance and T2DM.

The metabolic regulating enzyme AMP-activated protein kinase (AMPK) is the therapeutic target of several anti-diabetic agents, such as metformin [37] and adiponectin [38]. It is activated by phosphorylation via either the serine/threonine kinase 11 (a.k.a. liver kinase B1), or the calcium/calmodulin protein kinase kinase beta (CaMKKβ) pathway [39]. The anti-diabetic actions of hepatic AMPK activation are attributed to attenuation of lipogenesis and gluconeogenesis, as well as promotion of lipid oxidation and glycolysis [40]. Additionally, activation of hepatic AMPK has been reported to inhibit Foxo1 activity [41], which results in reduced hepatic ER stress, and to alleviate hepatic steatosis and insulin resistance [42,43]. Furthermore, prior clinical studies have shown that low serum concentrations of $25(OH)D_3$ are independently associated with liver steatosis [44].

Meanwhile, hypovitaminosis D has been proposed to be a causative factor of NAFLD [45]. Although liver steatosis is related to progression of hepatic insulin resistance, no specific lipid has been reported to be both necessary and sufficient for development of liver steatosis. NAFLD appears to involve the accumulation of a variety of lipids, and the levels of multiple lipids can be used as markers of insulin resistance status [46]. Excessive accumulation of certain lipids, such as diacylglycerol and acyl CoA, may interfere with glucose generation and has been linked to risk of hepatic insulin resistance [46–49]. Because hepatic glucose production is tightly regulated by the availability of the enzymes PEPCK and G6PK, and further modulated by the availability of fructose-1,6-bisphosphatase and pyruvate carboxylase [50], down-regulation of these enzymes might reduce abnormal gluconeogenesis in T2DM and ameliorate hepatic insulin resistance.

The research summarized above has led us to propose that vitamin D bioavailability may affect hepatic lipogenesis and gluconeogenesis, and if so, vitamin D supplementation may be used to modulate hepatic insulin resistance and thus reduce T2DM severity. Mechanistically, such effects may be mediated by various vitamin D-regulated pathways, such as AMPK-calmodulin and/or Akt/Notch signaling, as well as through indirect effects on ER stress. In light of the aforementioned negative association between vitamin D status and severity of NAFLD, a well-recognized risk factor for insulin resistance and T2DM, we are now investigating the direct effects of vitamin D on hepatic lipid and glucose production. We obtained preliminary data recently indicating that, at high dosages, calcitriol (the active hormonal metabolite of vitamin D) can ameliorate abnormal hepatic lipid and glucose metabolism in both *in vitro* (1–10 nM in HepG2 cells) and *in vivo* (0.5–2.5 mg/kg for 2 days in db/db mice) models of insulin resistance without any signs of toxicity (unpublished data). Furthermore, we conducted mechanistic experiments showing that increases in cytosolic calcitriol in HepG2 cells activated Ca^{2+}/CaMKKβ/AMPK pathways, and that the activation of these pathways contributed to calcitriol's lipid and glucose regulatory effects.

The involvement of AMPK signaling in calcitriol-mediated metabolic effects is not surprising given that AMPK is the therapeutic target of anti-diabetic medications (e.g., metformin) [37], as well as a target of the obesity regulating endogenous hormone adiponectin, which acts to alleviate insulin resistance [38]. Further study is needed to corroborate these findings, which suggest that calcitriol, when at above-physiological plasma concentrations, can reduce hepatic triglyceride accumulation and glucose output, at least in part, through activation of Ca^{2+}/CaMKKβ/AMPK signaling under insulin-resistant conditions. Importantly, unlike its inactive metabolic precursor cholecalciferol (vitamin D_3), calcitriol does not accumulate in adipose tissue or exert long-lasting effects. Thus, the potential for toxicity that exists with cholecalciferol is much less of a concern with calcitriol, owing to its rapid onset and offset of action. If confirmed, these preliminary data may provide an avenue to supporting the use of vitamin D, at least, as an adjuvant for the management of insulin resistance, NAFLD, and T2DM. Figure 2 is a summary that proposes the direct action of vitamin D in regulating hepatic triglyceride and glucose metabolism.

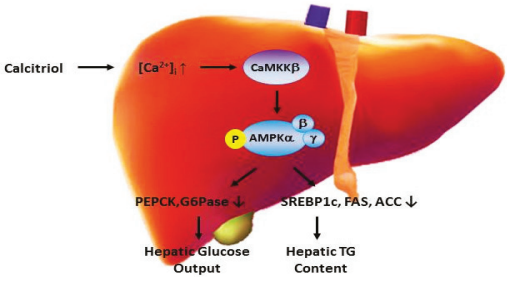

Figure 2. Model of active vitamin D regulation of hepatic triglyceride accumulation and glucose output in a diabetic state.

5. Vitamin D and Pancreatic Islet Function

Emerging data from physiological and genetic studies indicate that islet dysfunction and loss of beta-cell mass are the key determinants of whether an insulin-resistant state will progress to frank hyperglycemia/hyperlipidemia and diabetes; insulin resistance alone is insufficient to predict T2DM [51,52]. High circulating concentrations of glucose and fatty acids in diabetic states are attributed to loss of islet function and mass due to glucolipotoxicity, a process involving oxidative stress, ER stress, and inflammation [53]. Hence, the development of therapeutic agents that can protect islets from glucolipotoxicity could provide a much needed mode of improving the management of T2DM.

Several signaling pathways have been reported to play critical roles in insulin secretion as well as beta-cell growth and survival. For example, activation of Akt is closely associated with beta-cell survival [54–56] and promotes compensatory beta-cell growth in the insulin-resistant state [57]. Akt activation-induced phosphorylation inhibits GSK3 and Foxo1 which, in turn, reverses the toxic effects of glucose and fatty acids on beta-cells [58,59]. In addition, fatty acid-induced ER stress in islets has been linked with decreased Akt activity, and with activation of c-Jun NH_2-terminal kinase (JNK), which, ultimately, contributes to beta-cell apoptosis [60]. These findings lend support to the notion that Akt is a promising target for preservation of islet function and cell mass. In this context, recent studies have demonstrated that angiotensin (1–7), an active component of the renin-angiotensin system (RAS), modulates insulin resistance in skeletal muscle cells by way of Akt signaling pathway activation [61]. Activation of Akt/JNK pathways is also involved in angiotensin (1–7)-mediated modulation of palmitate-induced islet endothelial cell apoptosis [62]. Interestingly, it has been shown that glucagon-like peptide-1 and angiotensin II can prevent glucolipotoxicity-induced apoptosis in pancreatic beta cells additively through the insulin receptor substrate-2/phosphoinostide 3-kinase/Akt/FoxO1 signaling pathways [63]. Hence, it is plausible that other regulators, such as vitamin D (*vide supra*) might, like RAS ligands, exert islet-protective effects through activation of Akt signaling. Further work is needed to examine potential vitamin D-RAS axis interactions in the regulation of islet function and beta-cell survival.

Given the importance of hepatic and pancreatic islet functions in the pathogenesis of insulin resistance and T2DM, we are investigating promising factors and how they, alone or in combination, influence islet function under various pathological and physiological conditions. Of great interest in this context is the local pancreatic RAS and its potential involvement in islet function and survival in T2DM [64]. Previously, we demonstrated that inhibition of islet RAS signaling (*i.e.*, angiotensin II receptor type 1 activation) increased glucose-induced insulin secretion and improved glycemic control [65,66]. Mechanisms proposed for these effects include enhancement of intra-islet blood perfusion [67], reduction of oxidative stress [68], and improvement of the beta-cell proliferation-to-apoptosis balance [69]. Meanwhile, vitamin D is necessary for normal islet insulin secretion [9–11] and it is also a negative endocrine regulator of the RAS, suppressing renal renin secretion [70,71]. Moreover, hypovitaminosis D leads to defective insulin secretion, reduced glucose homeostasis, and increased risk of T2DM in all age bands [72,73], whereas increased pancreatic islet RAS activity in hyperglycemia impairs islet function and survival under hyperglycemic conditions [74]. Given these vitamin D-RAS interactions in islet survival, we set out to examine the potential modulatory action of vitamin D on regulation of islet function and survival through suppression of pancreatic islet RAS [74,75].

Toward this aim, we examined in *ex vivo* experiments how calcitriol (bioactive vitamin D metabolite) affects RAS component expression in islets from normal control, hypovitaminosis D, and VDR knockout mice under physiological and high-glucose conditions. We found that high-glucose–induced upregulation of islet RAS component expression could be prevented and corrected by calcitriol; in corroboration, the VDR knockout mice exhibited overactive islet RAS, compared with that of wild-type mice [76]. On the other hand, the mice with diet-induced hypovitaminosis D developed impaired glucose tolerance and exhibited increased expression RAS components combined with reduced expression of islet function-related genes [76]. Interestingly, we

observed in a subsequent study that pharmacological renin inhibition (*i.e.*, aliskiren treatment) without correction of hypovitaminosis D reduced islet RAS hyperactivity, ameliorated islet dysfunction and insulin resistance, and improved glucose tolerance [77].

In summary, treating mice with hypovitaminosis D with RAS inhibitors, without correction of vitamin D deficiency, reduced islet RAS over-activity, ameliorated islet dysfunction and improved glucose tolerance, as predicted. These data indicate that suppression of RAS hyperactivity in a condition of hyperglycemia and hypovitaminosis D is protective against pancreatic islet dysfunction and the development of insulin resistance, thus improving glucose tolerance and glucose homeostasis. If corrected, these findings help to explain the protective role of RAS inhibition against T2DM, as previously reported, and support the use of RAS inhibitors for treating hypertension in people who have, or are at risk of, T2DM. Further work is needed to determine whether alleviation of hypovitaminosis D could work synergistically with RAS inhibition to improve T2DM-related metabolic impairments. In light of these findings, we conclude with Figure 3 that represents a proposed model of how vitamin D regulates pancreatic beta-cell function and survival.

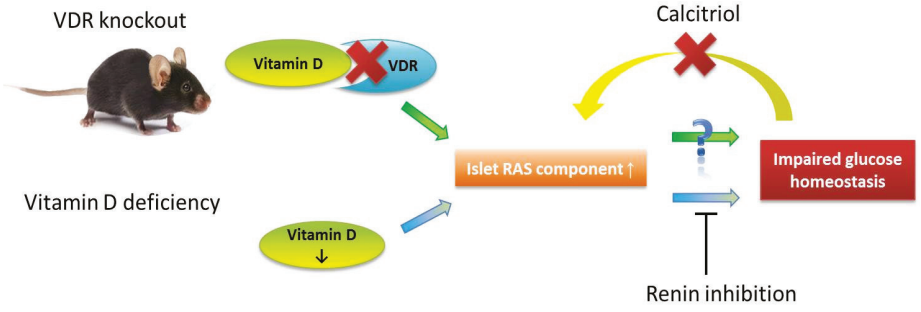

Figure 3. Model of vitamin D in the regulation of pancreatic islet beta-cell function and survival in a diabetic state.

6. Vitamin D and Pancreatic Islet Development

Recent advances in directed differentiation of pancreatic stem cells offer potential for the development of beta-cell replacement therapies for diabetes patients. Unfortunately, however, existing differentiation protocols are complex, time-consuming, and costly; thus there is a desperate need for alternative protocols that can be used to promote beta-cell proliferation, differentiation, and maturation [78]. In this regard, our group has established a system for the isolation and culture of pancreatic progenitor cells (PPCs) derived from human fetal pancreas tissue. These PPCs have a high capacity for proliferation and differentiation when cultured with an appropriate differentiation cocktail; their differentiation can be promoted by morphogens or growth factors related to human pancreatic development, e.g., secreted PDZ domain-containing protein 2 [79,80] and angiotensin II [81], as well as by vitamin A and vitamin D [82]. Vitamin A is a well-established modulator of beta-cell differentiation, and vitamin D is known to affect beta-cell insulin secretion; both vitamin A and vitamin D act through the RXR heterodimerization pathway (*vide supra*) [83–85].

Interestingly, we have demonstrated that the retinoic acid receptor, VDR, and RXR are expressed in first-trimester human fetal PPCs and that all-trans retinoic acid and calcitriol can (each alone) enhance PPC viability [82]. Although further investigations are warranted to elucidate the differentiation properties of PPCs and clarify the roles of vitamin A and vitamin D in islet development and beta-cell differentiation, these data suggest that vitamin A and vitamin D are involved in PPC development and thus should be considered in attempts to develop culture protocols for the development of insulin-secreting islet-like cell clusters (ICC) suitable for clinical transplantation into diabetic patients.

Given the common developmental origins of the liver and pancreas, it is also interesting to note that PPCs can be differentiated in a culture devoid of growth factors by using a microenvironment established by liver stromal cells (LSC) derived from human fetal liver [86]. Specifically, we demonstrated experimentally that a liver stromal cell-induced niche can enhance PPC differentiation into ICCs and enhance ICC functionality. This was the first to report that an LSC-induced niche can enhance ICC differentiation and functionality. Further modifications of the stroma microenvironment may offer an alternative, efficient and cost-effective approach to providing insulin-producing cells for clinical transplantation.

Finally, very recent studies implicated vitamin D in trans-generational risk of metabolic disease. A maternal high-fat diet during pregnancy and lactation was reported to affect hepatic fatty acid metabolism of rat offspring [87]. These animal findings have clinical relevance to obesity-associated diseases in humans and future studies are warranted to examine whether specific plasma fatty acid could be used as an early marker of hepatic dysregulation so as to assist in the identification of offspring that might have a risk of increased adiposity in adulthood. In fact, hypovitaminosis D is closely related to obesity [88] and, in fact, obesity is a causal factor for reduction of serum 25(OH)D levels [89]. These findings suggest that low vitamin D availability due to excessive maternal body fat may contribute to trans-generational impairment of liver fatty acid metabolism [90].

7. Conclusions

Vitamin D might have dual anti-diabetic influences: (1) modulation of hepatic glucose and lipid metabolism; and (2) promotion of pancreatic islet function and survival. Vitamin D could ameliorate hepatic glucose and lipid metabolism abnormalities *in vitro* and *in vivo* through activation of Ca^{2+}/CaMKKβ/AMPK signaling. Furthermore, vitamin D might have a RAS-suppressing influence that may benefit beta-cell function. Taking into consideration some negative data from clinical trial studies, our preliminary results with demonstrated beneficial effects of vitamin D on hepatic and pancreatic functions await to be intensively investigated and validated. In addition, our study findings obtained from cellular and animal models should also be interpreted cautiously since they are not always readily translated into human. However, given the high worldwide prevalence of obesity, T2DM, and related cardio-metabolic sequela that are associated with high healthcare costs and socio-economic implications, the potential protective effects of vitamin D warrants further exploration; if confirmed, vitamin D supplementation may represent a promising, cost-effective preventative and therapeutic agent for the management of obesity-related insulin resistance and diabetes.

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Postprandial Effect of a High-Fat Meal on Endotoxemia in Arab Women with and without Insulin-Resistance-Related Diseases

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Abstract: This study determined the effects of a high-fat meal on circulating endotoxin and cardiometabolic indices in adult Arab women. The cohort consisted of 92 consenting Saudi women (18 non-diabetic (ND)) control subjects; Age 24.4 ± 7.9 year; body mass index (BMI) 22.2 ± 2.2 Kg/m^2), 24 overweight/obese (referred to as overweight-plus (overweight$^+$)) subjects (Age 32.0 ± 7.8 year; BMI 28.5 ± 1.5 Kg/m^2) and 50 type 2 diabetes mellitus (T2DM) patients (Age 41.5 ± 6.2 year; BMI 35.2 ± 7.7 Kg/m^2). All were given a high-fat meal (standardized meal: 75 g fat, 5 g carbohydrate, 6 g protein) after an overnight fast of 12–14 h. Anthropometrics were obtained and fasting blood glucose, lipids, and endotoxin were serially measured for four consecutive postprandial hours. Endotoxin levels were significantly elevated prior to a high-fat meal in the overweight$^+$ and T2DM than the controls ($p < 0.05$). Furthermore, the postprandial cardiometabolic changes led to a more detrimental risk profile in T2DM subjects than other groups, with serial changes most notable in glucose, triglycerides, high density lipoprotein-cholesterol (HDL-cholesterol), and insulin levels (p-values < 0.05). The same single meal given to subjects with different metabolic states had varying impacts on cardiometabolic health. Endotoxemia is exacerbated by a high-fat meal in Arab subjects with T2DM, accompanied by a parallel increase in cardiometabolic risk profile, suggesting disparity in disease pathogenesis of those with or without T2DM through the altered cardiometabolic risk profile rather than variance in metabolic endotoxinaemia with a high-fat meal.

Keywords: endotoxin; type 2 diabetes mellitus; Arab women; high fat meal

1. Introduction

The nutritional transition and the rapid urbanization in the Middle East has introduced energy-dense refined carbohydrates and increased saturated fat intake [1]. This transition has paralleled the increase in lifestyle-related chronic diseases such as obesity and type 2 diabetes mellitus (T2DM) [2,3].

Whilst obesity represents the single most influential risk factor for T2DM, weight gain itself is a result of a complex interaction between genetic, epigenetic, and environmental factors. Amongst the latter, a carbohydrate-rich high-fat diet can quickly drive the increased obesity mediated T2DM [4]. The major metabolic consequence of a high-fat diet is the negative effect on insulin action, where the regulatory mechanisms of body weight become impaired through lipotoxic effects as well as the increased low grade chronic systemic inflammatory response [5,6].

Previous models of diet-induced and genetic obesity has shown that adipose tissue presents an important source of pro-inflammatory adipocytokines, such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) during weight gain [7,8]. These adipocytokines can have a simultaneous dual impact leading to insulin resistance through activation of the pro-inflammatory mechanisms as well as a direct impact on insulin signaling capacity [9]. As such, the development of the insulin resistance through a sustained energy dense diet promotes hyperinsulinaemic conditions, coupled with increased adipose tissue and ectopic fat accumulation [10].

The impact of a high-fat diet at the molecular level remains to be fully understood. However, postprandial lipidaemia has emerged as a potential candidate to promote an inflammatory response. Studies have shown that the ingestion of a single high-fat meal can mediate systemic increases of a wide range of inflammatory factors with noted activation of nuclear factor-κB (NF-κB) in leukocytes [11–14], a key transcription factor in the inflammatory cascade that regulates the transcription of numerous pro-inflammatory cytokines and adipocytokines [15,16]. To date, the cause of these postprandial inflammatory events remains poorly understood. One potential candidate factor is bacterial endotoxin (lipopolysaccharide (LPS)), a potent inflammatory bacterial antigen that is present in large quantities in the human gut [17]. Endotoxin circulates in the blood of healthy human subjects at low concentrations (between 1 and 200 pg/mL) [18]. However, clinical studies have implicated gut-derived endotoxin as a "primary insult" that activates the inflammatory state, contributing to metabolic disease, with cross sectional data showing elevated systemic endotoxin levels in obesity, T2DM, coronary artery disease and fatty liver disease with the ability to be influenced by changes in diet [19–23].

The aim of this study was therefore to determine the influence of a high-fat meal on changes in circulating endotoxin and whether this is altered in different metabolic disease states amongst Arab adult women.

2. Experimental Section

The study comprised of three groups of subjects: non-T2DM, lean subjects (control; Body Mass Index (BMI) 22.2 ± 2.2 Kg/m^2; n = 18), overweight/obese (referred to as overweight-plus (overweight$^+$)) subjects (overweight$^+$ 28.5 ± 1.5 Kg/m^2) subjects (n = 24), and patients with early onset of T2DM (BMI: 35.2 ± 7.7 Kg/m^2; n = 50). All subjects were Saudi pre-menopausal women, randomly selected from different primary care centers (PCCs) of Riyadh, Saudi Arabia, nonsmokers, with a normal resting electrocardiogram (ECG) and blood pressure, and with no history of vascular disease. In addition, subjects with known long-standing diabetes and/or receiving anti-diabetic medication, those with fasting glucose levels > 11 mmol/L, or with fasting triglycerides levels > 4 mmol/L were excluded from the study. Ethical approval was granted by the Ethics Committee of King Saud University (No. 10-173), Riyadh, Kingdom of Saudi Arabia, prior to the commencement of the research and all patients gave written consent.

Screening fasting blood tests at baseline were performed to qualify subjects for the study and to assess glucose control as well as lipid profiles. In addition, all subjects had their weight, height, waist and hip circumferences measured. Weight (in kilograms) was measured in light clothing to

the nearest 0.1 kg. Height was measured using a digital stadiometer to the nearest centimeter. Waist circumference was measured at the level of the iliac crest at the end of normal respiration, and hip was measured at the widest circumference around the buttocks using measuring tape. BMI, as well as waist-to-hip ratios (WHR), were calculated. Blood samples were taken from the right or left antecubital vein in the sitting position. Blood pressure was checked with a blood pressure monitor on the left arm using a standard protocol. All subjects (n = 92) with and without T2DM were given a high-fat meal (standardized meal: 75 g fat, 5 g carbohydrate, 6 g protein per m^2 body surface area corresponding to 700 Kcal/m^2 [24]) after an overnight fast of 12–14 h.

2.1. In Vivo Assessment of the Biochemical Profile

Blood samples were drawn via cannula at baseline (0 h) and postprandially (1, 2, 3, and 4 h), and endotoxin and lipid levels were measured. Serum glucose and lipid profile were measured routinely using a glucose oxidase method in an autoanalyzer (Konelab, Espoo, Finland). Serum-free insulin concentrations were determined by electro-chemiluminescence method (COBAS-E-411; Roche Diagnostics, Mannheim, Germany). Homeostasis model assessment for insulin resistance (HOMA-IR) was then calculated for all patients using the HOMA formula:

$$\text{HOMA-IR} = \text{fasting insulin (mU/L)} \times \text{fasting plasma glucose (mmol/L)}/22.5 \text{ [25]}. \quad (1)$$

2.2. Analysis of Circulating Endotoxin

Serum endotoxin was analyzed using a commercially available QCL-1000 LAL End Point Assay (Lonza, Allendale, NJ, USA). The assay, and the values given by the manufacturer for intra-assay coefficient of variation (CV) (3.9% $\pm$ 0.46%) and inter-assay CV (9.6% $\pm$ 0.75%), have been validated as detailed previously by this research group [16].

2.3. Statistical Analysis

Data were analyzed using SPSS version 16.5 (SPSS, Chicago, IL, USA). All continuous variables were presented as mean $\pm$ standard deviation and were normalized prior to parametric analyses. For comparison between groups, Analysis of Variance (ANOVA) with Tukey *post-hoc* analysis and Kruskal-Wallis (for triglycerides) were used. For comparison overtime, repeated measures ANOVA and Friedman's two-way analysis of variance (for triglycerides, insulin, HOMA-IR and endotoxin) were used. For associations between endotoxin and variables of interest postprandial, Spearman bivariate correlations were utilized. Significance was set at $p < 0.05$.

3. Results

The clinical and metabolic characteristics were determined (Table 1) according to groups. Subjects with T2DM had a mean duration of diabetes of 2.04 years. Furthermore, it noted that there were significant differences in age and BMI across the cohort (Table 1). As expected, the T2DM group also had the highest anthropometric indices (BMI, waist and hip circumferences as well as WHR) than the overweight$^+$ group and controls, with the overweight$^+$ group being significantly higher than the controls in all indices as well. As anticipated, subjects with T2DM had significantly higher serum glucose levels (7.9 $\pm$ 2.73 mmol/L) than the overweight$^+$ (4.7 $\pm$ 0.41 mmol/L, $p < 0.01$) and control subjects (4.81 $\pm$ 0.86 mmol/L, $p < 0.001$). The subjects with T2DM also had significantly higher serum triglycerides (1.9 $\pm$ 1.0 mmol/L), total cholesterol (5.4 $\pm$ 1.07 mmol/L) and low density lipoprotein-cholesterol (LDL-cholesterol) (3.66 $\pm$ 0.8 mmol/L), as well as the lowest in mean high density lipoprotein-cholesterol (HDL-cholesterol) (0.96 $\pm$ 0.21 mmol/L) (all $p < 0.001$). Serum glucose and lipid levels of the overweight$^+$ and control groups were not significantly different from one another (Table 1).

Table 1. Clinical and metabolic characteristics of subjects according to group.

	Control	Overweight^{+}	T2DM	*p*-Value
N	18	24	50	
Age (years)	24.4 ± 7.9	32.0 ± 7.8 *	41.5 ± 6.2 *$^{!}$	<0.001
T2DM Duration (years)	–	–	2.04 (0–9)	
BMI (Kg/m^2)	22.2 ± 2.2	28.5 ± 1.5 *	35.2 ± 7.7 *$^{!}$	<0.001
Waist (cm)	80.6 ± 7.2	95.8 ± 7.4 *	112.3 ± 13.4 *$^{!}$	<0.001
Hip (cm)	98.7 ± 7.3	109.7 ± 5.0 *	117.1 ± 11.6 *$^{!}$	<0.001
WHR	0.8 ± 0.05	0.9 ± 0.05 *	1.0 ± 0.07 *$^{!}$	<0.001
Glucose (mmol/L)	4.8 ± 0.9	4.7 ± 0.4	7.9 ± 2.7 *$^{!}$	<0.001
LDL-Cholesterol (mmol/L)	2.8 ± 0.6	2.8 ± 0.7	3.7 ± 0.8 *$^{!}$	<0.001
Triglycerides (mmol/L) #	1.0 ± 0.4	1.3 ± 0.8	1.9 ± 1.0 *$^{!}$	0.001
Total Cholesterol (mmol/L)	4.2 ± 0.7	4.5 ± 01.0	5.4 ± 1.1 *$^{!}$	0.003
HDL-Cholesterol (mmol/L)	1.3 ± 0.2	1.1 ± 0.4	0.96 ± 0.2 *$^{!}$	<0.001

Data presented as mean ± standard error; # denotes non-Gaussian distribution; *p*-values at extreme right denotes over-all significance according to group; "*" denotes significance as compared with control subjects; "$^{!}$" denotes significance as compared with overweight^{+} group; "–" denotes absence of T2DM in subjects; *p*-value significant at < 0.05. Analysis of Variance (ANOVA) with Tukey *post-hoc* analysis and Kruskal-Wallis (for triglycerides) tests were used (T2DM: Type 2 diabetes Mellitus; BMI: Body mass index; WHR: Waist hip ratio; LDL: Low density lipoprotein; HDL: High density lipoprotein).

3.1. Effects of High-Fat Meal in Different Groups

Blood samples were taken over time following a high-fat meal to assess changes in metabolic indices and endotoxin changes in the three cohorts. In the T2DM group, mean glucose level was highest at 0 h and lowest after 4 h, which was significantly lower than other hours with a noted stepwise reduction over time (Table 2). In both the control and overweight^{+} groups, no significant changes in glucose were noted over time (Table 2). In contrast to glucose levels, the triglyceride levels for all groups followed an increasing trend over time. The triglyceride levels in the subjects with T2DM was highest postprandially between 3 and 4 h, and was significantly higher than hours 0–2 ($p < 0.01$). In the overweight^{+} group, triglyceride levels were highest at 3 h than 4 h, with a stepwise increase from baseline over time until 3 h. Comparing all groups, the subjects with T2DM had significantly higher mean triglyceride levels as compared with either the overweight^{+} or control groups ($p < 0.01$). No significant changes were noted with total cholesterol over time.

In the subjects with T2DM, HDL-cholesterol was lowest after 4 h and was significantly lower than hours 0–3 ($p < 0.01$) with, again, a stepwise reduction in HDL-cholesterol level (Table 2). Similarly, the HDL-cholesterol levels in the overweight^{+} group were lowest at hour 4 than the previous hours 0–2 ($p < 0.01$). In contrast, the HDL-cholesterol levels of the control group were significantly higher than both the T2DM and overweight^{+} groups ($p < 0.01$).

In the T2DM group, LDL-cholesterol levels at baseline were significantly higher than hours 1–4 ($p < 0.05$). In the overweight^{+} group, LDL-cholesterol levels at baseline were significantly higher than hours 2–4 ($p < 0.05$). In the control group, LDL-cholesterol levels at baseline was significantly lower than hours 1 and 2 ($p < 0.05$) returning to baseline levels after that. T2DM had significantly higher LDL-cholesterol levels than either the control or overweight^{+} group.

Table 2. Metabolic changes pre- and post-high-fat meal.

	0 h	1 h	2 h	3 h	4 h
GLUCOSE (mmol/L)					
T2DM (N = 50)	7.9 ± 2.7	7.8 ± 2.5	7.5 ± 2.5 *	7.29 ± 2.7 *$^{!§}$	7.0 ± 2.8 *$^{!§†}$
Overweight^{+} (N = 24)	4.7 ± 0.4	4.6 ± 0.4	4.6 ± 0.4	4.6 ± 0.39	4.63 ± 0.7
Control (N = 18)	4.8 ± 0.86	5.1 ± 2.3	5.02 ± 1.7	4.76 ± 1.51	4.79 ± 1.6
TRIGLYCERIDES (mmol/L) #					
T2DM (N = 50)	1.9 ± 1.0	1.8 ± 0.7	2.4 ± 0.9 *$^{!}$	2.7 ± 1.1 *$^{!§}$	2.7 ± 1.3 *$^{!§}$
Overweight^{+} (N = 24)	1.3 ± 0.8	1.4 ± 0.8	1.7 ± 0.9 *$^{!}$	2.0 ± 1.1 *$^{!§}$	1.9 ± 1.3 *$^{!§}$
Control (N = 18)	1.0 ± 0.4	1.2 ± 0.6	1.4 ± 0.9	1.44 ± 0.91	1.54 ± 1.0
TOTAL CHOLESTEROL (mmol/L)					
T2DM (N = 50)	5.4 ± 1.1	5.3 ± 1.0	5.4 ± 1.1	5.3 ± 1.1	5.4 ± 1.1
Overweight^{+} (N = 24)	4.5 ± 1.0	4.5 ± 0.9	4.4 ± 0.8	4.4 ± 0.8	4.4 ± 1.0
Control (N = 18)	4.2 ± 0.7	4.1 ± 0.7	4.1 ± 0.6	4.1 ± 0.6	4.2 ± 0.7
HDL-CHOLESTEROL (mmol/L)					
T2DM (N = 50)	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2 *$^{!}$	0.89 ± 0.2 *$^{!§†}$
Overweight^{+} (N = 24)	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.4 *$^{!}$	1.1 ± 0.4 *$^{!§}$
Control (N = 18)	1.3 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
LDL-CHOLESTEROL (mmol/L)					
T2DM (N = 50)	3.7 ± 0.8	3.6 ± 0.8	3.4 ± 0.9 *	3.2 ± 0.9 *	3.3 ± 0.9 *
Overweight^{+} (N = 24)	2.8 ± 0.7	2.7 ± 0.6	2.5 ± 0.6 *	2.4 ± 0.6 *	2.4 ± 0.6 *
Control (N = 18)	2.7 ± 0.6	2.6 ± 0.6*	2.6 ± 0.6 *	2.6 ± 0.6	2.7 ± 0.6
Endotoxin (EU/mL)					
T2DM (N = 50)	3.4 ± 0.8	3.0 ± 0.8	3.4 ± 0.9 $^{!}$	3.5 ± 0.9 $^{!}$	3.6 ± 0.9 $^{!}$
Overweight^{+} (N = 24)	3.0 ± 0.5	2.9 ± 1.4	3.5 ± 0.9	3.8 ± 1.6	3.5 ± 1.9
Control (N = 18)	1.5 ± 0.1	1.8 ± 0.1 *	1.7 ± 0.8 *	1.9 ± 0.2 *	2.1 ± 0.2 *
Insulin (IU/mL) #					
T2DM (N = 50)	11.7 ± 5.5	21.9 ± 17.7 *	19.3 ± 12.6 *	16.2 ± 12.3 *	14.3 ± 8.2 *
Overweight^{+} (N = 24)	5.0 ± 3.4	15.6 ± 16.2 *	16.9 ± 16.0 *	13.1 ± 8.0 *	10.3 ± 5.3 *
Control (N = 18)	5.8 ± 0.64	10.3 ± 1.7	9.6 ± 3.4	8.2 ± 1.6	10.2 ± 2.6
HOMA-IR #					
T2DM (N = 50)	3.7 ± 2.0	8.7 ± 12.0 *‡	6.6 ± 6.0 *‡	5.6 ± 5.4 ‡	3.9 ± 2.4
Overweight^{+} (N = 24)	1.12 ± 0.7	3.5 ± 4.1 *	3.9 ± 4.1 *	2.8 ± 1.9 *	2.1 ± 1.2
Control (N = 18)	1.3 ± 0.2	1.9 ± 0.4	2.1 ± 0.7 *	1.9 ± 0.4	2.2 ± 0.6

# denotes Non-Gaussian distribution; * denotes significance compared with 0 hour; $^{!}$ denotes significance compared with 1; § denotes significance compared with 2; † denotes significance compared with 3; ‡ denotes significance compared with 4; p significant at $\leq$0.05. Repeated measures ANOVA and Friedman's two-way analysis of variance (for triglycerides, insulin, HOMA-IR and endotoxin) were the tests used (T2DM: Type 2 diabetes Mellitus; Overweight^{+}: overweight/obese subjects referred to as overweight-plus; LDL: Low density lipoprotein; HDL: High density lipoprotein; HOMA-IR: Homeostasis model assessment for insulin resistance; ANOVA: analysis of Variance).

The highest endotoxin levels were noted at hour 4 in all groups and was statistically significant compared with hour 1 in the T2DM group and baseline in the control group ($p < 0.01$). No significant changes were observed in the overweight^{+} group. Both the T2DM and overweight^{+} groups had significantly higher endotoxin levels than the control subjects. Insulin levels were also noted to change over time in the subjects with T2DM, with the highest peak insulin levels noted at hours 1–2, lowest at hours 3–4, and baseline fasted insulin levels being significantly lower as compared to insulin levels across time ($p < 0.01$). This similar pattern was observed in the overweight^{+} and control groups, with the baseline noting the lowest insulin levels over time. Between groups, the insulin level in the T2DM group was significantly higher than either the overweight^{+} or control group as expected ($p < 0.01$).

Lastly, in both the T2DM and overweight$^+$ groups, HOMA-IR was lowest at baseline at the fourth hour with the T2DM group showing the highest mean HOMA-IR post 1 h feed and the overweight$^+$ group at hour 2. No significant changes in HOMA-IR were observed in the control group over time. Between groups, the HOMA-IR in subjects with T2DM was significantly higher than those of the overweight$^+$ and control groups ($p < 0.01$).

3.2. Associations of Metabolic Parameters to Endotoxin after a High-Fat Meal

In all subjects, endotoxin was positively associated with LDL cholesterol ($R = 0.38$; $p < 0.05$) and this was observed 3 h after a high-fat meal. In the subjects with T2DM, endotoxin was significantly associated with triglycerides at 3 and 4 h postprandial (R values 0.52 and 0.50; $p < 0.05$, respectively). In the overweight$^+$ subjects, endotoxin was highly associated with triglycerides ($R = 0.63$; $p < 0.05$) and total cholesterol ($R = 0.71$; $p < 0.05$) at baseline. Whilst in the control subjects no associations were observed at baseline or over time (Table 3).

4. Discussion

The present results affirm and extend our knowledge on the impact of a high-fat meal in different metabolic states. Specifically, circulating endotoxin levels were significantly raised during a high-fat meal in overweight$^+$ and T2DM metabolic states, with the impact of cardiometabolic changes imposing a more detrimental risk profile in T2DM subjects than either the overweight$^+$ or the non-diabetic lean control subjects as assessed by glucose, triglycerides, insulin and HOMA-IR and lower HDL postprandially. The results also highlighted that the same single meal given to subjects in different metabolic states had a different impact on cardiometabolic health. As such, daily repetition of this type of meal could have a much more damaging effect in the subjects with T2DM, closely followed by the overweight$^+$ subjects; whilst lean and non-diabetic subjects appear to better handle the insult of a high-fat meal, metabolically.

Despite the impact of the meal it was also identified that prior to the high-fat meal, the presence of sub-chronic inflammation was already apparent, with circulating endotoxin being highest in subjects with T2DM, affirming prior studies that suggest that endotoxin levels are altered in the presence of insulin-resistant diseases [16,19–22,26]. Previous studies have also shown that a high-fat meal, regardless of the individual's metabolic status, can induce inflammatory changes [27,28]. In this study, circulating endotoxin, a potential mediator of a low grade chronic inflammatory response, was comparably raised in both the overweight$^+$ and T2DM states, unlike previous studies in South Asians [26], suggesting that Arabs in the overweight$^+$ state may at least be at even higher metabolic risk, which could help to explain the country's current higher metabolic disease per capita.

Elevated endotoxin levels amongst Middle-Eastern patients with T2DM are also consistent across ethnic groups, such as Africans [29], Chinese [30], Caucasians [16,31], and South Asians [22,23]. Whilst endotoxin is seen as an important mediator of sub-clinical inflammation, Piya and colleagues have suggested that, ultimately, the gut flora may act as an essential determinant of the sub-chronic inflammation induced by obesity and T2DM, and that endotoxin may act as a systemic insult that triggers the inflammatory cascade [32]. In Saudi women, a high-fat meal given to both overweight+ and T2DM groups increased endotoxin levels from a higher baseline in these groups without a clear difference between them, in contrast to previous studies in South Asians [26]. Animal studies have shown that continuous infusion of endotoxin increases gut permeability, as does high-fat dietary feeding, therefore, one possible explanation for the difference could be due to the dietary differences in fat consumption that the obese subjects eat in Saudi Arabia as compared in UK [33].

Table 3. Bivariate associations between lipids, glucose and endotoxin.

ALL SUBJECTS																									
Glucose					**Triglycerides**					**Total Cholesterol**					**HDL-Cholesterol**					**LDL-Cholesterol**				
0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
−0.17	0.04	0.13	0.08	0.00	−0.13	0.10	0.14	0.22	0.20	0.16	−0.08	0.00	−0.07	−0.21	−0.16	0.15	−0.01	−0.08	−0.06	0.22	−0.06	0.05	0.38	−0.12
T2DM SUBJECTS (*N* = 50)																								
−0.20	0.23	0.29	0.23	0.08	−0.12	0.32	0.26	0.52	0.50	0.18	0.04	0.02	0.0	−0.08	−0.28	-0.15	−0.14	−0.30	−0.05	0.30	0.02	0.005	−0.06	−0.19
OVERWEIGHT^{+} SUBJECTS (*N* = 24)																								
0.23	0.14	0.08	0.0	−0.18	0.63	0.04	0.08	0.26	0.25	0.71	−0.08	0.10	−0.13	−0.33	−0.13	0.41	0.13	−0.07	−0.18	0.36	0.14	0.43	0.64	0.28
CONTROL SUBJECTS (*N* = 18)																								
−0.36	0.15	0.22	0.24	0.14	−0.20	0.16	0.30	−0.14	−0.17	–	0.01	0.14	−0.05	−0.26	−0.28	−0.20	−0.39	−0.16	−0.31	−0.02	0.08	0.24	0.07	−0.09

Data presented as coefficient (*R*); bold and red denotes significance at $p < 0.05$. Spearman correlation tests were used (T2DM: Type 2 diabetes Mellitus; Overweight^{+}: overweight/obese subjects referred to as overweight-plus; Control Subjects: Non-daibetic lean individiuals; LDL: Low density lipoprotein; HDL: High density lipoprotein). "–" denotes absence of T2DM in subjects.

Animal studies involving *ob/ob* and *db/db* mice have demonstrated a leaky gut which is considered to be related to the impact insulin resistance on gut endothelium [33]. As such, our overweight$^+$ and T2DM subjects may have a more frequent snack habit, which consequentially affects how they subsequently handled the high-fat meal, which could be different from the white Caucasians given such a diet. Furthermore, ethnicity may clearly impact on individual sub-clinical inflammatory risk. Previous studies have shown that endotoxin can be stratified by gender and ethnicity; therefore, these data could help explain why different ethnicities have a variable cardiometabolic risk due to post-feeding increases in endotoxin [21].

The current study also considered the impact of a high-fat meal on elevating glucose and cholesterol levels, both considered important in the development of coronary artery disease, coupled with obesity and T2DM. The changes observed postprandially in the lipid profile and insulin in this study, resulted in lipidaemia regardless of their metabolic status and this is consistent with previous studies [26,34–36]. Prior analysis of the effects of a high saturated fat meal in other studies indicates that lipidaemia can mediate deleterious changes at the proteome and genome levels producing a pro-coagulant state [37]. Furthermore, it appears from other studies that triglycerides show the most dynamic changes during the postprandial phase compared to other lipids, regardless of the individual's metabolic status, although it appears dependent on the type of fat diet used [38]. Clinically, this is relevant. As observed in the present and other studies, higher postprandial hypertriglyceridemia and hyperlipidemia are observed amongst subjects with T2DM than their healthier counterparts [39]. Therefore, dietary strategies defining the type of dietary fat for patients, to lower saturated fat content, appears important to lead to better management of the insulin response and increased fat oxidation, both important determinants of cardiometabolic risk in overweight and T2DM patients [40].

The different cardiometabolic risk factor changes in pre- and post-feeding showed that the T2DM group had significantly higher glucose, triglycerides, insulin, and HOMA-IR, as well as significantly lower HDL-cholesterol throughout the high-fat challenge as compared with overweight$^+$ and control subjects. Whilst these significant differences were expected, since the baseline levels of T2DM subjects were already higher than other groups, the persistence of dysmetabolism in the T2DM group suggests that already deranged baseline glycemic and lipid parameters could be exacerbated further following a fat intake [41,42]. The postprandial increments in glucose and lipids observed confirms previous studies with women and even adolescents with T2DM [43–45] and also reaffirms the requirement to effectively manage postprandial glucose and lipid response in T2DM subjects through diet intervention to lower cardiovascular disease risk [42,46,47].

The authors acknowledge the significant variation in the mean age of the groups and this may have limited the present findings, taking into account the evidence that postprandial lipemia may be associated with age [48]. Nevertheless, comparisons were done mostly within groups and not independent of one another whilst observing for postprandial similarities and differences in patterns according to groups. Furthermore, the small and unequal sample size per group may have affected the few significant associations elicited out of a hundred possible correlations conducted, suggesting that these associations need to be further validated using a larger cohort per group.

5. Conclusions

In conclusion, our findings highlight that subjects with increased adiposity and or T2DM are at increased cardiometabolic risk given a high-fat meal, but particularly so in the subjects with T2DM. Furthermore, irrespective of their diabetic status, endotoxin levels, postprandial, were higher than control subjects. As such, the findings suggest that the disparity in lipidaemia, as opposed to endotoxin, post-meal, may exacerbate the pathogenesis of cardiovascular disease in Arab women in the long-term on this type of diet. Therefore, dietary interventions that can reduce the glycaemic and lipidomic response, as well as improving the gut microbiota for better gut barrier function, appears important in Saudi patients who have increased weight gain or a T2DM status.

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

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Review

Insulin-Sensitizing Effects of Omega-3 Fatty Acids: Lost in Translation?

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Abstract: Omega-3 polyunsaturated fatty acids (*n*-3 PUFA) of marine origin, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have been long studied for their therapeutic potential in the context of type 2 diabetes, insulin resistance, and glucose homeostasis. Glaring discordance between observations in animal and human studies precludes, to date, any practical application of *n*-3 PUFA as nutritional therapeutics against insulin resistance in humans. Our objective in this review is to summarize current knowledge and provide an up-to-date commentary on the therapeutic value of EPA and DHA supplementation for improving insulin sensitivity in humans. We also sought to discuss potential mechanisms of *n*-3 PUFA action in target tissues, in specific skeletal muscle, based on our recent work, as well as in liver and adipose tissue. We conducted a literature search to include all preclinical and clinical studies performed within the last two years and to comment on representative studies published earlier. Recent studies support a growing consensus that there are beneficial effects of *n*-3 PUFA on insulin sensitivity in rodents. Observational studies in humans are encouraging, however, the vast majority of human intervention studies fail to demonstrate the benefit of *n*-3 PUFA in type 2 diabetes or insulin-resistant non-diabetic people. Nevertheless, there are still several unanswered questions regarding the potential impact of *n*-3 PUFA on metabolic function in humans.

Keywords: insulin resistance; EPA; DHA; *n*-3 PUFA; mitochondria; muscle

1. Introduction

There is tremendous interest in the health benefits of omega-3 polyunsaturated fatty acids (*n*-3 PUFA), which include the essential fatty acid α-linolenic acid (ALA) and longer-chain fatty acids, eicosapentaenoic (EPA) and docosahexaenoic (DHA), derived from marine organisms. There is an extensive body of literature dedicated to understanding, among the many chronic diseases that may benefit from dietary intake of *n*-3 PUFA, its therapeutic potential in the context of type 2 diabetes. Albeit the FDA supports the use of *n*-3 PUFA as a treatment for hypertriglyceridemia, and the Mediterranean diet as a preventive strategy for cardiovascular disease [1], there is not a clear consensus from human trials on the systematic use of *n*-3 PUFA supplements for people with insulin resistance or type 2 diabetes. Substantial inconsistencies exist between studies of humans compared to rodents. While most studies in rodents suggest a favorable effect of omega-3 fatty acids on glucose utilization and insulin sensitivity, human studies have been conflicting. Although some report improvement in insulin sensitivity with fish oil consumption, the majority of human studies do not recapitulate the findings. Prior review reports extensively covered this subject [2–5], however, a synopsis of more current knowledge is needed with greater attention to the reasons behind this apparent lack of translatability from rodents to humans. We, therefore, conducted a literature search of recently published studies within the last two years and extended the discussion to include representative studies published earlier. This review focuses on the influence of EPA and DHA on insulin sensitivity

and summarizes the outcomes of recent animal studies, human observational studies, and randomized clinical trials, while rendering further insight into mechanistic data on skeletal muscle metabolism and mitochondrial response to *n*-3 PUFA supplementation.

1.1. Insulin Resistance

Insulin resistance (IR) is an early metabolic abnormality in the course of obesity, metabolic syndrome, and type 2 diabetes. The prevalence of insulin resistance is high worldwide, with approximately 35% of US adults having insulin resistance [6]. Insulin stimulates skeletal muscle glucose uptake, and inhibits hepatic glucose production and adipose tissue lipolysis. In conditions of insulin resistance, these actions are impaired, leading to a vicious cycle of fasting or postprandial hyperglycemia, elevated free fatty acids, hyperinsulinemia, and pancreatic β-cell dysfunction [7]. Although IR is more evident in obesity [8,9], it is also noted in lean individuals with a high genetic component [10–12], and has been related to a constellation of abnormalities, such as skeletal muscle mitochondrial dysfunction [13], ectopic lipid accumulation [14], liver steatosis [15], inflammation [16], oxidative stress [17], and aging [18]. The major culprit, however, for the high prevalence of insulin resistance is a positive energy balance, derived from an excessive food consumption of low nutritional value and a sedentary lifestyle [19,20].

1.2. n-3 PUFA

EPA and DHA are very long chain polyunsaturated fatty acids (VLC *n*-3 PUFA) that incorporate into cell membranes following consumption of fatty fish, such as salmon, herring, mackerel, tuna, and sardines. Their biosynthesis is limited in the human body by relatively inefficient desaturase and elongase enzymes that convert ALA into VLC *n*-3 PUFA. Established properties of *n*-3 PUFA are their anti-inflammatory action and lowering effect on triglycerides [21], and they have been used as complimentary strategies in coronary heart disease [22,23] and retinopathy [24], while there is extensive research on improving cognitive disorders [25], telomeric aging [26], and cancer progression [27]. Much controversy still exists on their effect on glucose metabolism and insulin action. Mechanistic information about their action is still under investigation, however, their pleiotropic effects are purported to be related to their chemical structure. The long tail of double bonds confers some unique chemical and physical properties; they are extremely flexible molecules with rapid transitions between a large number of conformers, indicating a key property for entering into the binding pockets of proteins [28]. In addition, their cis bonds limit the ability of the fatty acids to be closely packed when incorporated into cell membranes, driving the formation of lipid domains, such as lipid rafts [29], which then enable or inhibit the interaction with signaling proteins and regulation of downstream pathways [29–31]. Thus *n*-3 PUFA are believed to affect membrane fluidity and also modulate expression of genes via regulation of transcription factors related to energy supply, cell cycle regulation, and cell differentiation [32].

2. Preclinical Studies of *n*-3 PUFA and Insulin Resistance

2.1. High Calorie Diet-Induced Insulin Resistance

There are a plethora of studies on mouse and rat models of insulin resistance following a high carbohydrate (HCD) or high fat (HFD) diet. Rodents fed a HCD or HFD increase their weight, serum, and liver triglycerides and become hyperinsulinemic with impaired glucose tolerance. In a recently published study in rats, HCD enriched with dietary DHA and EPA for 30 days could reverse these symptoms [33]. In this study, fish oil was supplemented as 2%, 5% and 7% of the fat intake. Interestingly, 2% supplementation was not effective in reversing insulin resistance, while 5% and 7% attenuated the HOMA-IR index and had a dose-dependent effect on increasing the expression of genes related to hepatic lipid β-oxidation and decreasing the expression of genes related to hepatic *de novo* lipogenesis (SREBP-1c, ChREBP). This indicates enhancement of insulin action in the liver and

a protective effect of *n*-3 PUFA in the course of development of insulin resistance. Nevertheless, the effective doses of *n*-3 PUFA were beyond the recommended levels of the American Heart Association for adult humans, which range up to 4 g/day (~4.5% of fat intake) [34].

In support of the aforementioned study, we recently showed that HFD-induced insulin resistance in mice was partially prevented by substituting 3.4% kcal of saturated fat intake with *n*-3 PUFA [35]. Mice were fed a control diet (10% fat), HFD (60% fat,) or HFD plus EPA/DHA for 10 weeks. Body weight and fat mass increased similarly in HFD and HFD plus EPA/DHA groups, indicating that the protective effects of *n*-3 PUFA were not mediated by modifying adiposity; it has been reported however that *n*-3 PUFA supplementation might ameliorate body weight [36,37]. Following an oral glucose tolerance test, the EPA/DHA group maintained glucose levels similar to controls. Considering that the insulin response at 15 min was similarly increased for both HFD and *n*-3 PUFA, albeit measurements were not done for the whole 2-h duration, the beneficial effect in glycemia is likely to be attributable to greater insulin sensitivity. This is in consistency with other studies reporting greater insulin sensitivity with fish oil supplementation in a HFD background in mice [36,38,39] and rats [39].

In terms of insulin signaling and gene expression, we also showed that *n*-3 PUFA increased the mRNA expression of insulin-stimulated glucose transporter-4 (GLUT4), insulin receptor substrate-1 (IRS1) and glycogen synthase-1 (GYS1) in skeletal muscle, corroborating the finding of enhanced glucose utilization [35]. Consistent with these data, in the same model of HFD-fed mice, Lamping KG *et al.* demonstrated the superiority of fish oil compared to monounsaturated olive oil and *n*-6-enriched fish oil, in restoring basal glucose levels, glucose tolerance, and insulin signaling, including AKT phosphorylation [40]. Similarly in rats, Lionetti *et al.* showed that a HFD rich in fish oil (40% fat) compared to a HFD rich in lard (40% fat) for six weeks, also increased GLUT-4 and IRS1 transcripts expression in muscle, concomitant with improvements in insulin sensitivity, thus yielding some mechanistic explanation of the *n*-3 PUFA effect on skeletal muscle. The rescue of insulin signaling by *n*-3 PUFA, which is initially suppressed by a HFD, has been suggested to be tissue specific, with differential effects on muscle, liver, and adipose tissue [40]. Most studies, however, confirm the beneficial effect of *n*-3 PUFA on muscle, involving insulin receptor (IR) density, IR and IRS1 phosphorylation, phosphatidyloinositol (PI) 3′-kinase activity, and GLUT-4 content [41]. Luo *et al.* also suggested a beneficial effect of DHA in HFD-fed mice on adipose tissue angiogenesis and insulin resistance, via the silent information regulator 1 (SIRT 1) pathway. SIRT1 belongs in a family of proteins which are purported to be involved in the regulation of glucose homeostasis and attenuate insulin resistance via reducing mitochondrial dysfunction [42].

We therefore conclude that observations recapitulated in recent and numerous prior studies [43–47] provide strong evidence that *n*-3 PUFA prevent the reduction in glucose tolerance and insulin sensitivity induced by a high fat diet background in rodents.

2.2. Muscle-Liver Glucoregulatory Axis

White P.J. *et al.* proposed a new model of omega-3 action mediated by its active metabolites, termed specialized proresolving mediators (SPM), which include resolvins (Rv), protectins (PD), and maresins (MaR). These bioactive lipid metabolites are, in general, associated with the resolution of obesity-linked inflammation and insulin resistance in high-fat fed mice [48]. The authors suggested that protectin DX (PDX), produced via lipoxygenation of DHA, is responsible for activating a myokine–liver glucoregulatory axis, through stimulation of IL-6 release from skeletal muscle. IL-6 is thought to regulate hepatic glucose production via induction of STAT3 phosphorylation which in turn suppresses expression of gluconeogenic genes in the liver, including peroxisome proliferator-activated receptor γ coactivator-1α (PPARGCo1a), phosphoenolpyruvate carboxykinase (Pck1), and glucose-6-phosphatase (G6pc). Insulin sensitivity was assessed by a hyperinsulinemic-euglycemic clamp with a paired 'lipid infusion plus PDX or vehicle', and 'saline infusion plus PDX or vehicle'. Interestingly PDX improved both peripheral and hepatic insulin action in WT mice, while results were abrogated in IL-6 null mice.

In diabetic db/db mice, PDX lowered glycemia without affecting insulin concentration, indicating the superiority of its effect on hepatic insulin action inhibiting gluconeogenesis [49]. PDX also displayed higher phosphorylation of the AMP-activated protein kinase (AMPK) in muscle, which is considered as another potent mechanism mediating the effects of omega-3 fatty acids on insulin sensitivity [50]. This study suggested that the therapeutic benefits of *n*-3 PUFA might be due, in part, to the distinct actions of their bioactive metabolites, which may further mediate cross-organ communication.

2.3. Anti-Inflammatory Effects

Chronic macrophage-mediated inflammation is considered a hallmark of insulin resistance, and omega-3 fatty acids are purported to exhibit anti-inflammatory effects. A landmark study by Oh *et al.* reported that the G-protein-coupled receptor 120 (GPR120) expressed predominantly in mature adipocytes, macrophages, and hepatic stellate cells, functions as an omega-3 fatty acid receptor/sensor. By signaling through GPR 120, *n*-3 PUFA inhibit both TLR and TNF-α inflammatory signaling pathways and likely mediate M1–M2 macrophage polarization by decreasing the expression of inflammatory genes (IL-6, TNF-a, MCP-1, IL-1b, iNOS, CD11c) and increasing the expression of anti-inflammatory genes in adipose tissue (IL-10, MGL1, YM-1, Clec7a, MMR). GPR120 KO mice were glucose intolerant, hyperinsulinemic, and displayed skeletal muscle and hepatic insulin resistance, which was not ameliorated by *n*-3 PUFA supplementation. On the contrary, HFD-insulin-resistant WT mice supplemented with *n*-3 PUFA improved overall insulin sensitivity through mitigation of inflammation and increased adipose tissue glucose uptake [47]. Other studies also explored the prevention or reversal of IR in HFD-induced obese mice via modulation of adipose tissue inflammation, with a reported increase in anti-inflammatory cytokines (adiponectin) in plasma [51].

2.4. Hepatoprotection

Additional liver lipidomic profiling by Oh *et al.* revealed that *n*-3 PUFA ameliorated HFD-induced steatosis, supporting the view that omega-3 treatment can reverse non-alcoholic fatty liver disease (NAFLD) mediated in large part by GPR 120 [47]. Hepatoprotection and prevention of NAFLD, with repression of hepatic stellate cell activation and fibrogenesis in the liver, was also reported in mice fed HFD-enriched with fish oil for six weeks [39] and 12 weeks [52]. This was observed in parallel with a decrease in liver oxidative stress and was associated with improvements in HOMA-IR, adiponectin plasma levels, and overall insulin sensitivity. Liu X *et al.* also confirmed that EPA supplementation alone was efficacious in suppressing body fat accumulation and alleviated insulin resistance measured by OGTT in a HFD, HCD background. EPA alone also efficiently alleviated hepatic steatosis by modulating the suppression of adipocytokines (adiponectin) and inflammatory cytokines (TNFa, IL-6), and suppressed SREBP-1c-mediated lipogenesis while enhancing lipid β-oxidation (Liu, Xue *et al.* 2013). In line with these observations, a recent review by Delarue *et al.* concluded that LC-*n*-3 PUFA decrease liver steatosis, but do not reverse already established histologic features of non-alcoholic stehatohepatitis (NASH) [53].

A study by Poudyal *et al.* measured the independent effects of ALA, EPA, and DHA in high calorie-fed rats and all *n*-3 PUFA individually reduced inflammation in both heart and liver, as well as reducing cardiac fibrosis and hepatic steatosis. These effects were associated with complete suppression of stearoyl-CoA desaturase 1 (Scd-1) activity, a marker implicated in cardiovascular disease, insulin resistance, and obesity [37].

2.5. Oxidative Stress

Amelioration of oxidative stress in various tissues was also reported in a spontaneously hypertensive obese rat model (SHROB) of the metabolic syndrome. Treatment with EPA/DHA for 13 weeks increased the activity of antioxidant enzymes in erythrocytes, abdominal fat, and kidneys, and lowered the plasma C-reactive protein (CRP) inflammation marker. Of note, the magnitude of the activation varied depending on different EPA/DHA ratios, with 1:2 having the strongest effect on

oxidative stress *versus* 1:1 and 2:1 [54]. In contrast, we did not find any effect of fish oil on skeletal muscle antioxidant enzymes activity from HFD-mice, including sodium dismutase (SOD1) and catalase, although catalase mRNA expression was found to be significantly higher in the fish oil-supplemented group [35].

2.6. n-6:n-3 PUFA Ratio

A study demonstrating the anti-inflammatory properties of *n*-3 PUFA via suppression of toll-like receptor 4 (TLR4) activation in muscle reported improvements in inflammatory markers (TNFα, CRP, IL-6) and insulin resistance as measured from oral glucose and insulin tolerance tests (OGTT and ITT) [55]. These effects, however, were determined by a specific ratio of *n*-6:*n*-3 PUFA of 1:1, and were not replicated by a ratio of 4:1, suggesting that a balance among polyunsaturated fatty acids might be a more important contributor to metabolic health, rather than absolute levels of *n*-3 PUFA, as reported previously [56,57]. Other studies in non-rodent animals also attempted to delineate the metabolic importance of the *n*-6:*n*-3 PUFA ratio. Duan *et al.* fed pigs with either a 1:1, 2.5:1, 5:1, and 10:1 *n*-6:*n*-3 PUFA ratio and showed that the 5:1 ratio led to the highest growth performance, while the 1:1 diet led to increased muscle mass and lowest adipose tissue mass, with concomitant changes in inflammatory markers [58]. Although the metabolic significance of the *n*-6:*n*-3 PUFA ratio is still under investigation, considering the very high average ratio of the Western diet, 16–17:1, it is likely that an optimized *n*-6:*n*-3 PUFA ratio may exert beneficial effects on lipid metabolism, inflammation status, and other metabolic pathways.

2.7. n-3 PUFA Role in Aging

Aging is purported to be associated with a higher inflammatory status and insulin resistance. We, therefore, fed young and old mice with normal chow enriched with either EPA or DHA and performed large-scale proteomics and mRNA sequencing analysis from muscle tissue samples. Among the top metabolic pathways affected by EPA and DHA, was downregulation of the acute phase response signaling pathway, suggesting that *n*-3 PUFA ameliorated the inflammatory status in the muscle of old mice compared to young controls [32]. In addition, EPA supplementation enhanced muscle protein quality in the old mice by reducing carbamylation, a post-translational modification known to be driven by inflammation [32,59]. Further, we detected no differences in glucose tolerance or insulin sensitivity, indicating that *n*-3 PUFA might be beneficial only when a certain level of metabolic dysfunction is established, but not when insulin sensitivity is within a normal range. Aging is also associated with reduced mitochondrial function, which is implicated in the pathogenesis of insulin resistance [13]. Discussion of the effect of *n*-3 PUFA on mitochondrial function in the context of aging is reported under the section, "EPA and DHA Effect on Skeletal Muscle Metabolism".

2.8. Preclinical Studies in Primates

The majority of reports in rodents suggest beneficial effects of VLC-*n*-3-PUFA on insulin sensitivity and glucose tolerance; however, data in humans are ambiguous. An interesting report comes from a study in non-human primates, male rhesus monkeys, which develop features of the metabolic syndrome under a HCD. Supplementation with 4 g/day EPA + DHA for six months prevented fructose-induced hypertriglyceridemia and insulin resistance, as assessed by intravenous glucose tolerance testing. The area under the curve (AUC) for glucose remained fairly stable, however, both groups exhibited increased insulin concentrations, suggesting β-cell compensation for insulin resistance. Fish oil mitigated the hyperinsulinemia, as well as the increase in leptin and apolipoprotein E, without any changes in adiponectin, body weight, and fat mass [60]. Since primates provide a better model of human metabolism than rodents, these observations bring further interest in the translational value of the findings.

3. Human Studies of *n*-3 PUFA and Insulin Resistance

3.1. Observational Studies in Adults

Most observational studies assess the relationship of omega-3 fatty acids levels, measured in plasma and erythrocyte membranes, with the prevalence of metabolic syndrome (MetS). Due to the nature of the cross-sectional studies and the large cohorts of participants involved, the most common and feasible method used to assess insulin resistance is the homeostasis model assessment (HOMA-IR), derived from fasting glucose and insulin concentrations. Consequently, HOMA-IR is not a representative measurement of insulin sensitivity in postprandial conditions and could lead to erroneous assumptions. The majority of observational studies reflect a favorable effect of *n*-3 PUFA on insulin action. Most of the studies found an inverse association between *n*-3 PUFA content and the index of insulin resistance [61–63], even in populations with a high intake of fish oil and a low risk for MetS and diabetes, like the Alaska Eskimos [64,65], indicating that *n*-3 PUFA could assist in the prevention or treatment of insulin resistance in humans. Of note, the associations were significant even after adjusting for age, gender, BMI, and ethnicity [62]. Biosynthesis of EPA in the body measured by the Δ5 desaturase index was associated with insulin resistance in normoglycemic, but not in glucose intolerant, adults in a Cree Canadian population [63]. In a Korean population, *n*-3 PUFA were not predictors of increased risk for metabolic syndrome [66]. Nevertheless, in a prospective study in the same population of Korean healthy adults, ages 40–69 years, who were followed for three years for MetS and CVD outcomes, the consumption of fish oil was associated with a lower risk for MetS among men, but not among women [67]. In a recently performed observational study from the National Heart, Lung, and Blood Institute (NHLBI) Family Heart study, in 4941 participants, there was no association of fish oil consumption with MetS in a large U.S. population [68]. Consecutively, there appear to be geographic differences in the responsiveness to *n*-3 PUFA, potentially related to environmental factors and/or genetic variations, as well as usual dietary habits and lifestyles.

3.2. Observational Studies in Children and Adolescents

Observational studies in children render similar results to studies in adults, showing an inverse association of omega-3 fatty acids with HOMA-IR [69,70], lower plasma levels of EPA in obese children with IR compared with obese children without IR [70], and beneficial associations between *n*-3 PUFA and lipid profile [71]. Conflicting results come from a Danish study, where DHA was associated with a poor metabolic profile [72]. However, in obese children, DHA content had an inverse association with BMI [73], suggesting that overall dietary management, including omega-3 fatty acids, could be an important tool in managing childhood obesity.

4. *n*-3 PUFA in Human Clinical Trials

4.1. Meta-Analyses

A summary of recent RCTs is shown on Table 1. There was no change in insulin sensitivity in 10 out of 13 identified randomized, double-blind, placebo-controlled trials. In consistency, a systematic meta-analysis (of 11 RCTs published until October 2010, with $n = 618$ participants) concluded that *n*-3 PUFA consumption did not affect insulin sensitivity [74]. However in a sensitivity analysis by measures of IS, a positive relationship was found in the HOMA sub-group compared to the control, but not in the QUICKI sub-group. This observation is difficult to interpret, because HOMA and QUICKI are equivalent and crude measurements of IS, yet it cannot be disregarded. Another systematic review, which examined the effect of EPA and DHA on metabolic syndrome risk factors, concluded that there are no clear effects on metabolic syndrome markers, except for an improvement in blood pressure and the well-established hypotriglyceridemic effect. Interestingly, lower doses of *n*-3 PUFA were associated with further benefits of reducing pro-atherogenic small dense particles (sdLDL), whereas greater doses ($\geqslant$3 g/day) were associated with increases in LDL cholesterol [75].

Table 1. Summary of human clinical trials in non-diabetic individuals.

NON DIABETIC					
Study	**Objective/Participants**	**Dose**	**Duration**	**Method**	**Effect on Insulin Sensitivity**
RCTs double-blinded, placebo-controlled					
Lalia AZ *et al.* 2015 [76]	IR, overweight, $n_t = 14$, $n_c = 11$	3.9 g/day	6 months	Pancreatic Clamp	No change
Root M *et al.* 2013 [77]	Effects of FO on vascular health and arterial stiffness. Overweight BMI>23 kg/m^2, young adults 18–30 y, $n_t = 30$, $n_c = 27$	1.7 g/day	1 month	FBG	No change
Spencer M *et al.* 2013 [78]	Effect of FO on adipose tissue inflammation, obese, IR with MetS $n_t = 19$, $n_c = 14$	4 g/day	3 months	IVGTT	No change
Derosa G *et al.* 2012 [79]	Dyslipidemic patients $n_t = 78$, $n_c = 79$	3 g/day	6 months	Clamp	No change
Mohammadi E *et al.* 2012 [80]	Iranian, PCOS women, overweight or obese, 20–35 y, $n_t = 32$, $n_c = 32$	1.2 g/day	2 months	HOMA-IR	Improved
Kelly DS *et al.* 2012 [81]	Hypertriglyceridemic men $n = 14$–17/group	3 g/day DHA	3 months	HOMA-IR, Matsuda index	No change
Toktam F *et al.* 2010 [82]	Schizophrenia or bipolar disorder $n_t = 20$, $n_c = 21$	<1 g/day	1.5 months	HOMA-IR	No change
Fakhrzadeh H *et al.* 2010 [83]	Effects of FO on serum lipid profile of elderly Iranians, ⩾65 y, $n = 124$	300 mg/day	6 months	HOMA-IR	No change
Ahren B *et al.* 2009 [84]	Young 20–37 y: lean BMI 20–26 kg/m^2 $n = 12$, obese BMI 29–35 kg/m^2 $n = 10$. Old 50–65 y: lean $n = 16$, obese $n = 11$ Crossover design	3g + CLA 3 g/day, control 6 g/day	3 months	Mixed meal	No change/Decreased in older obese
Browning LM *et al.* 2007 [85]	Overweight women with inflammatory phenotype. Top quartile $n = 12$, low quartile $n = 18$ Crossover design with 1 month washout.	4.2 g/day	3 months	OGTT	Improved top tertile
Giacco *et al.* 2007 [86]	Healthy men and women, $n = 162$	3.6 g/day	3 months	IVGTT	No change
Griffin MD *et al.* 2006 [87]	Effect of *n*-6:*n*-3 ratio with 4 diets between 5:1 and 3:1, men and postmenopausal women from the OPTILIP study, 45–70 y, $n = 258$	6% Kcals	6 months	HOMA-IR and QUICKI	No change

Table 1. *Cont.*

NON DIABETIC					
Study	**Objective/Participants**	**Dose**	**Duration**	**Method**	**Effect on Insulin Sensitivity**
RCTs singe-blinded, placebo-controlled					
Oh, PC *et al.* 2014 [88]	Hypertriglyceridemia, $n = 44$ in each group	1 g/day, 2 g/day, 4 g/day	2 months	QUICKI	No change
Rajkumar H *et al.* 2014 [89]	Healthy, 40–60 y, overweight, 4 groups: placebo, omega-3 fatty acids, probiotic, omega-3 fatty acids +probiotic. $n = 15$ per group	<1 g/day	1.5 month	HOMA-IR	Improved
Ramel *et al.* 2008 [90]	Effect of energy-restricted diets with FO, $n = 324$, European, young 20–40 y, overweight or obese, 4 diets: no seafood $n_c = 80$, lean fish 150 g cod 3/week $n = 80$, fatty fish 3/week $n = 84$, fish oil EPA/DHA capsules $n = 80$	1.3 g/day	2 months	HOMA-IR	Improved
Soares de Oliveira Carvalho AP *et al.* 2014 [91]	Effect of hypocaloric diet plus FO in women with MetS, 30–45 y, $n_t = 15$, $n_c = 15$	0.41 g/day	3 months	HOMA-IR	Improved
Stephens FB *et al.* 2014 [92]	Healthy young men: saline *vs.* 10% *n*-6 PUFA, *vs.* 2:1 *n*-6:*n*-3 PUFA, $n = 6$	total 20 g	acute IV lipid infusion	Clamp	Improved
RCTs—no placebo					
Yamamoto T *et al.* 2014 [93]	Hyperlipidemic patients, 54–84 y, $n_t = 31$, $n_c = 29$	900 mg/day EPA	3–6 months	HOMA-IR	Improved
Yamamoto T *et al.* 2014 [93]	Patients undergoing cardiac surgery, 54–86 y, $n_t = 10$, $n_c = 12$	1.8 g/day EPA	1 month	HOMA-IR	No change
Tsitouras PD *et al.* 2008 [94]	6 men and 6 women over 60 y	720 g fatty fish/week + 15 mL sardine oil/day	2 months	Octerotide insulin suppression testing	Improved
Meta-analyses of RCTs					
Akinkuolie *et al.* 2011 [74]	11 RCTS	0.138–11 g/day	6 weeks–6 months	HOMA-IR/QUICKI/ Clamp/IVGTT	No change

n-3 PUFA: omega-3 polyunsaturated fatty acids; *n*-6 PUFA: omega-6 polyunsaturated fatty acids; FO: fish oil; n_t: number of participants in the treatment group; n_c: number of participants in the control group; y: years of age; IR: insulin resistance; FBG: fasting blood glucose; IVTT: intravenous tolerance test; HOMA-IR: homeostasis model assessment of insulin resistance.

4.2. Limitations in Translating Results from Animal to Human Studies

As it will be discussed in the following sections, human intervention studies, in their majority, have failed to recapitulate the protective effect of EPA and DHA on glucose metabolism and insulin sensitivity that have been observed in rodents. The reasons for this incongruence between humans and animals, besides the conceivable genetic and phenotypic interspecies differences, are unclear. However, the majority of studies in rodents have been preventive, whereas human studies examine the curative potential of omega-3 fatty acids to reverse an already established metabolic dysfunction. This methodological discordance is compounded by the use of relatively crude indices of insulin sensitivity, including QUICKI or HOMA-IR, compared to more sensitive tools such as OGTT, mixed-meal challenge, and the hyperinsulinemic-euglycemic clamp. Furthermore, there is a huge variety in the dosage and duration of *n*-3 PUFA administered in both animal and human studies, precluding easily unified conclusions (Table 2). With the majority of human studies being observational, and a paucity of randomized, double-blinded, placebo-controlled trials of adequate size and power, any conclusions for the systematic use of *n*-3 PUFA in regulating human metabolic health await further verification.

Table 2. Confounders in translating animal–human studies.

	Etiology for Discrepancies in Studies of *n*-3 PUFA Action
1	Dosage of *n*-3 PUFA
2	Ratio EPA:DHA
3	Source of fish oil
4	Absorption and Bioavailability
5	Duration of intervention
6	Type of placebo
7	*n*-6:*n*-3 ratio
8	Type of cohort (young *vs.* old, NG *vs.* IFG *vs.* IR *vs.* DM, lean *vs.* overweight *vs.* obese, race, inflammatory status, comorbidities, usual dietary habits, and lifestyle)
9	Method to assess IS (hyperinsulinemic euglycemic clamp *vs.* HOMA-IR *vs.* OGTT)
10	Preventive study or therapeutic
11	Size and power of study

n-3 PUFA: omega-3 polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; NG: normal glucose; IFG: impaired fasting glucose; IR: insulin resistance; DM: type 2 diabetes mellitus; HOMA-IR: homeostasis model assessment of insulin resistance; OGTT: oral glucose tolerance test.

4.3. Representative RCTs

In order to conclusively evaluate the long-term effects of *n*-3 PUFA on insulin sensitivity in humans, it is essential to conduct carefully controlled studies with sufficient duration of supplementation and gold-standard measurements of outcomes. While most intervention studies vary from one to three months, our team recently conducted a six-month randomized, double blind, placebo-controlled trial in 31 insulin-resistant and overweight or obese adults who received 3.9 g/day EPA+DHA [76]. This was considered an adequate timeframe for the long-term effects of a pharmacologically relevant dose of *n*-3 PUFA. The study was carefully conducted with the use of a pancreatic hyperinsulinemic-euglycemic clamp and, consistent with previous reports, there was no effect of *n*-3 PUFA on whole body insulin sensitivity in the fasted postabsorptive state. We also observed no changes in postprandial glucose disposal and insulin secretion following a mixed-meal challenge. Furthermore, plasma inflammatory markers did not change with the intervention in consistency with a few other studies [76–78]. There is one more RCT by Derosa *et al.*, with a robust study design of 3 g/day *n*-3 PUFA supplementation for six months and use of a hyperinsulinemic-euglycemic

clamp to measure insulin sensitivity. The study included a large cohort of patients with combined dyslipidemia (control group n = 79, treatment group n = 78), who also received an oral fat load to assess inflammation response. The results demonstrated that, although there was an improvement in insulin sensitivity in the *n*-3 PUFA group, the effect was not significantly different from the controls. In addition, participants received a controlled-energy diet with 600 Kcal daily energy deficit, and were encouraged to increase their physical activity, both of which might have confounded the actual *n*-3 PUFA effect. In this metabolically unhealthy cohort, *n*-3 PUFA resulted in an improved response to oral fat load with higher HDL levels, and lower triglycerides, inflammatory markers (CRP, TNFa, IL-6), cell adhesion molecules, and other atherosclerotic risk factors, including metalloproteinases 2 and 9 [79].

Further, two double-blind, placebo-controlled RCTs, which used the intravenous glucose tolerance test (IVGTT) to assess insulin sensitivity following a three-month intervention with an adequate dose of *n*-3 PUFA, also reported no change in insulin sensitivity (S_I). Giacco *et al.* randomly assigned isoenergetic diets rich in monounsaturated (MUFA) or saturated fat (SFA) to 162 healthy Caucasian adults and further subdivided the groups to receive 3.6 g/day fish oil, or a placebo. None of the four diets affected insulin sensitivity (S_I) and the insulin disposition index or BMI. When the investigators accounted for higher (>4.85) or lower (<4.85) *n*-6:*n*-3 PUFA ratio, the same results persisted [86]. The second study by Spencer *et al.* focused on the effect of *n*-3 PUFA on adipose tissue in adults with impaired glucose tolerance, impaired fasting glucose, or metabolic syndrome. Supplementation with 4 g/day EPA and DHA reduced adipose (but not muscle) macrophages and plasma macrophage chemoattractant protein 1 (MCP-1) and increased capillary density. Despite these favorable effects on indices of chronic inflammation, there was no change in SI [78].

Two additional large RCTs of six-month *n*-3 PUFA supplementation showed no change in insulin sensitivity based on HOMA-IR [83,87]. In specific, a cohort of 124 elderly Iranian men received 300 mg/day of EPA and DHA, or a placebo, and showed reduced serum triglycerides, however, no other effects were detected in fasting blood sample variables and the index of insulin resistance [83]. Of note, this was a relatively small dose of *n*-3 PUFA, and albeit the time frame was adequate to achieve a cumulative effect in lowering triglycerides, it was less likely to have a prominent effect on insulin sensitivity compared to other studies where a larger dose of *n*-3 PUFA was used. The OPTILIP study in UK provided five diets of different *n*-6:*n*-3 PUFA ratios in 258 middle-aged and older adults who were at risk of ischemic heart disease. The aim of the study was to identify an optimal ratio for dietary recommendations to reduce cardiovascular risk. Therefore it included a food-based intervention rather than use of supplements, posing some limitations in standardizing the actual *n*-3 PUFA intake. The ratios varied from 10:1 (controls) to 5:1, 3:1 (EPA, DHA, ALA), 3:1 (ALA), and 3:1 (EPA, DHA). Decreasing the *n*-6:*n*-3 PUFA did not influence measures of insulin resistance, but confirmed the favorable effect in reducing triglycerides and proatherogenic sdLDL. Consecutively, the aforementioned studies provided evidence which does not support a long-term beneficial effect of *n*-3 PUFA in insulin sensitivity, despite measurable effects on cardiovascular risk factors and inflammation.

4.4. n-3 PUFA Effect and Inflammation

Inflammation is purported to be a leading mechanism in insulin resistance and metabolic disease. While most of the RCTs did not demonstrate a favorable effect of *n*-3 PUFA on insulin sensitivity, there are studies which reported improvement, as measured by HOMA-IR, and these studies had as a common denominator cohorts of participants with a high background inflammatory status. Browning *et al.*, using a cross over study design, divided 30 overweight or obese women into tertiles of inflammatory status based on concentrations of sialic acid. Following 4.2 g/day of three-month *n*-3 PUFA supplementation and an oral glucose tolerance test, women in the top tertile for inflammation demonstrated improved insulin sensitivity, whereas those at the reference lowest tertile did not [85]. The women in the raised inflammatory status also had higher BMI and insulin resistance, as well as fibrinogen and CRP. However, the fact that the improvement in insulin sensitivity was not accompanied

by significant changes in CRP, TNF-α, IL-6, α-1 anti-chymotrypsin, plasminogen activator inhibitor-1 (PAI-1), and α-1 acid glycoprotein (AGP), is suggestive that these plasma inflammatory markers are crude measurements of the *n*-3 PUFA anti-inflammatory effect or that the effect is mediated via other pathophysiological pathways. This corroborates the null findings from RCTs on relatively healthy adults with either moderate hypertriglyceridemia (Skulas-Ray, 2011 #5679) [95], or chronic lifestyle stress (Muldoon, 2016 #5670) [96] and low dietary EPA and DHA intake. Despite the beneficial effect on triglycerides, low, moderate, or pharmacological doses of *n*-3 PUFA had no effect on serum inflammatory markers (CRP and IL-6) *versus* a placebo.

Interesting data were also reported from patients with chronic renal failure under hemodialysis, where systemic inflammation and nutritional deficiency of proteins and essential fatty acids are prevalent and predictive of poor clinical outcomes. Supplementation of 2.4 g/day for two months led to significant improvement in HOMA-IR, ferritin levels, and inflammatory markers of TNF-α, IL-6 and hs-CRP with no significant changes in anthropometric characteristics [97]. In a second cross-sectional study of 111 hemodialysis patients, adequate intake of *n*-3 PUFA and a lower ratio of *n*-6:*n*-3 PUFA were associated with a higher total skeletal muscle mass [98]. Since skeletal muscle is the main target of insulin action, this study is relevant to understanding the effect of *n*-3 PUFA on insulin sensitivity and underscores the potential anabolic properties of *n*-3 PUFA in humans with an increased inflammatory status. Similarly, in patients with cancer cachexia from pancreatic cancer, it is well recognized that proinflammatory cytokines, such as TNF-α and IL-6, can induce the cachectic state, and acute-phase protein inflammatory response strongly predicts poor prognosis. Twenty-six patients with a median age of 56 years received an escalated high dose of 6 g/day EPA, which reversed the rate of weight change from a median loss of 2 kg/month to a median gain of 0.5 kg/month. This significant weight stabilization was achieved during the first month of the intervention and maintained for the remaining two months. The effect was also associated with the downregulation of proinflammatory cytokines, although other mechanisms at the level of gene expression and transcription cannot be excluded [99].

Another sensitive population group who could benefit from *n*-3 PUFA intervention are women with polycystic ovary syndrome (PCOS). PCOS is a common endocrinology disorder associated with obesity, a high degree of insulin resistance, and increased risk factors for diabetes and cardiovascular disease. An intervention in young Iranian PCOS women for two months of 1.2 g/day *n*-3 PUFA led to 21.8% improvement in HOMA-IR, compared to a placebo. This was in parallel with significant increase in serum adiponectin, an adipose tissue-derived cytokine with anti-atherogenic and anti-inflammatory effects. In addition, there were favorable effects in the lipid profile, with improvement in total and LDL cholesterol, but no significant changes in HDL or CRP [80].

The regulation of adipokine secretion by *n*-3 PUFA, including adiponectin and leptin, has been previously reviewed [100] and verified in recent studies [93]. A compelling study comes from Yamamoto *et al.*, who used EPA administration preoperatively for one month in patients undergoing cardiac surgery, a procedure liable to initiate an acute stress inflammatory response. Although they did not find any changes in insulin sensitivity or cardiac adverse events, pre-EPA treatment significantly decreased the neutrophil–lymphocytre ratio (NLR), mediated possibly by increases in adiponectin levels. This resulted in a decreased risk for post-operative infection through enhanced cell-mediated immunity, and underscored a whole new field for novel applications of *n*-3 PUFA in the clinical setting.

In this regard, formation of specialized pro-resolving mediators (SPM) from *n*-3 PUFA, termed resolvins, protectins, and maresins, may underlie some of the beneficial effects attributed to their precursors, EPA and DHA. Inflammatory response as a protective mechanism should be self-limited. However, in conditions where inflammation persists (acute phase response or chronic), SPM have the intriguing ability to selectively stimulate resolution of the inflammation without immune suppresion. Consequtively SPMs may be very effective from a therapeutic standpoint by terminating neutrophil recruitment and inflammatory cytokines release, and stimulating macrophage clearance and tissue regeneration [101,102]. Although there is an emerging body of evidence on their anti-inflammatory action and organ protective effects, as identified in the eye, kidney, lung, and periodontal tissue [103,104],

clinical studies in humans to address the potential therapeutic benefits of SPMs in insulin-sensitive tissues and in the context of insulin resistance and glucose tolerance are needed.

4.5. n-3 PUFA and Energy-Restricted Diets

Improvement in insulin sensitivity as measured by HOMA-IR was also noted in conditions of hypocaloric diets [90,91], which suggested that fish oil exerts positive effects on fasting insulin, and this was reported to be independent of weight loss or changes in plasma triacylglycerol and adiponectin concentrations [90]. Of note, these beneficial effects were seen with relatively low doses of *n*-3 PUFA (Table 1). There still remains skepticism about whether the effects of omega-3 fatty acids were confounded by the robust insulin-sensitizing effects of caloric restriction [105], or whether there is a positive interaction of *n*-3 PUFA with dietary and exercise modifications of energy intake and expenditure.

In this regard, *n*-3 PUFA have also been studied for their efficacy in weight-loss interventions. This is particularly relevant because adiposity is a great determinant of insulin resistance [106]. Nevertheless, results have not been unanimous among human studies, with previous reports showing reduced body fat and increased resting fat oxidation in healthy adults [107], and reduced trunk fat and adipocyte diameter in type 2 diabetes patients, without changes in insulin sensitivity as measured by an insulin clamp [108]. When coupled with energy restricted diets, there was no effect of *n*-3 PUFA on body fat of young athletes [109] or overweight women [110], but there was a report of greater weight loss and waist circumference reduction in overweight men [111]. When fish oil was added to exercise regimens, there was an independent effect on body fat reduction in overweight and obese adults [112], but no effect in lean young male volunteers [113]. Also, in severely obese women, aerobic exercise plus a very low-calorie diet plus 2.8 g/day *n*-3 PUFA, led to a greater reduction in BMI and hip circumference compared to exercise plus diet plus placebo. Although these studies did not assess insulin sensitivity, they could indicate a potential beneficial role of *n*-3 PUFA via reducing adiposity. The modest reduction in body fat or adipocyte size in cohorts of obese adults is not in contrast with their aforementioned anabolic effect on skeletal muscle, since previous reports have been supportive of a tissue-specific action of *n*-3 PUFA.

4.6. RCTs in Children

Clinical trials in children and adolescents with insulin resistance and obesity also rendered favorable outcomes, with general improvements in fasting glucose, insulin, triglycerides, BMI [114], HOMA-IR, TNFa, leptin, adiponectin [115], and blood pressure [116]. Nevertheless, more accurate measurements of insulin sensitivity in children and adolescent populations are missing and would be required in order to establish the therapeutic benefit of *n*-3 PUFA in this vulnerable population.

4.7. n-3 PUFA in Type 2 Diabetes

A brief overview of randomized clinical trials in type 2 diabetes patients is summarized in Table 3. It has been long proposed that omega-3 fatty acids do not provide beneficial effects on the glycemic control of patients with established type 2 diabetes [2,117]. Two meta-analyses, including 18 and 23 RCTs respectively, with large numbers of participants, were concordant in outcomes, describing a decrease in triglycerides, a potential increase in LDL, and no effect on glycemic control or fasting insulin from *n*-3 PUFA supplementation [2,117].

Table 3. Summary of human studies of the *n*-3 PUFA effect on Type 2 DM patients.

TYPE 2 DM					
Study	**Participants**	**Dose**	**Duration**	**Method**	**Effect on Insulin Sensitivity**
RCTs					
Farsi PF *et al.* 2014 [118]	Effect of FO on IS and NEFA, $n = 44$	4 g/day	2.5 months	HOMA-IR/QUICKI	Improved
Crochemore IC 2012 [119]	Effect of FO on IR and lipemia in obese women, $n = 41$, single-blind	2.5 g/day and 1.5 g/day	1 month	HOMA-IR/QUICKI	No change
Mostad IL *et al.* 2009 [120]	$n = 11$, crossover design	0.04 g/kg	acute lipid infusion	Clamp	No change
Mostad IL *et al.* 2008 [121]	Normotriglyceridemic without insulin treatment, $n_t = 12$, $n_c = 14$, placebo-controlled	5.9 g/day	9 weeks	Clamp	Decreased
Kabir M *et al.* 2007 [108]	Effect of FO on adiposity and atherogenic markers in women, $n = 27$	3 g/day	2 months	HOMA-IR/Clamp (in a subgroup of $n = 5$)	No change
Rasic-Milutinovic Z *et al.* 2007 [97]	Hemodialysis patients or chronic renal failure, $n = 35$	2.4 g/day	2 months	HOMA-IR	Improved
Mostad IL *et al.* 2006 [122]	Normotriglyceridemic, $n = 26$, double-blind controlled	5.9 g/day	1 week/9 weeks	Clamp	Decreased
Rivellese AA 1996 [123]	Hypertryglyceridemia, $n_t = 8$, $n_c = 8$, double-blind, placebo-controlled	2.7 g/day for 2 mon then 1.7 g/day for 4 mon	6 months	Clamp	No change
McManus *et al.* 1996 [124]	Well-controlled T2DM, $n = 11$, crossover design	~2.5 g/day	3 months	FSIGT	No change
Annuzi *et al.* 1991 [125]	NIDDM, male, $n = 8$, double-blind crossover design	10 g/day	0.5 month	Clamp	No change
Meta-analyses of RCTs					
Hartweg 2008 [117]	23 RCTs, $n = 1075$	3.5 g/day (mean)	9 weeks (mean)	FBG, FI	No change
Montori 2000 [2]	18 RCTs, $n = 823$	3–18 g/day	12 weeks (mean)	FBG	No change

FO: fish oil; n_t: number of participants in the treatment group; n_c: number of participants in the control group; IS: insulin sensitivity; IR: Insulin resistance; NEFA: non esterified fatty acids; FBG: fasting blood glucose; FI: fasting insulin; FSIGT: frequently sampled intravenous glucose tolerance test; HOMA-IR: homeostasis model assessment of insulin resistance.

In addition, meta-analyses of prospective studies had variable outcomes and reported no effect of *n*-3 PUFA on diabetes risk [126,127], beneficial effects in Asian populations [127–129], no associations among Europeans, and increased risk for incidence of diabetes in U.S. populations [128]. The observed differences between geographical regions could be a reflection of heterogeneous diets, including fish consumption, as well as racial and ethnic genetic differences. A more recent observational study by Lou DJ *et al.* [130] assessed the relationship of serum *n*-3 PUFA levels with IR and non-alcoholic fatty liver disease (NAFLD) in patients with type 2 diabetes, and reported that *n*-3 PUFA levels were significantly lower in T2DM and NAFLD and negatively correlated with HOMA-IR.

Among the most recent RCTs (Table 3), only one reported an improvement in HOMA-IR, with a concomitant decrease in NEFA following supplementation of 4 g/day for 2.5 months [118]. The majority of the studies which used the hyperinsulinemic-euglycemic clamp to assess insulin sensitivity, showed no beneficial effect of omega-3 fatty acids irrespective of duration and dosage [108,120,123,125]. There were, however, two randomized, double-blind, placebo-controlled studies by Mostad IL *et al.* which reported a moderate but significant deterioration of glycemia in subjects treated with fish oil. C-peptide responses also tended to be enhanced by fish oil, while an interesting effect was noticed in increased fat utilization compared to carbohydrate oxidation in the fasting state, following nine weeks of the intervention [121,122].

4.8. Preventive Versus Therapeutic Action of n-3 PUFA

Most animal studies are designed to prevent the effects of insulin resistance in a HFD or HCD background, while human studies are designed to reverse already established IR, an important distinction that may explain conflicting outcomes. An interesting approach to this notion was taken by conducting trials of an acute administration of lipid emulsions, enriched with omega-3 fatty acids *versus* placebo. Acute lipid infusions showed improvement in insulin sensitivity measured by a six-hour hyperinsulinemic-euglycemic clamp in six healthy young men [92]. In Type 2 DM patients, however, Mostad *et al.* used a four-hour lipid infusion with 0.04 g/kg *n*-3-PUFA enrichment and found no acute effect on insulin sensitivity. Also, neither insulin secretion, nor markers of oxidative stress, leptin, adiponectin, or energy expenditure, measured by indirect calorimetry, were affected [120]. Therefore, more trials are needed to verify the acute action of *n*-3 PUFA in insulin sensitivity and their potential role as preventive strategies for metabolic syndrome in humans.

Positive results were reported from a study with a preventive design by Delarue and colleagues. Insulin resistance was acutely induced in eight healthy, young, lean men with the use of dexamethasone for two days and participants were studied before and after three weeks of 1.8 g/day EPA plus DHA (6/day of fish oil) supplementation. The study demonstrated a 17% decrease in insulinemia as measured by the six-hour AUC following an oral glucose load. There was no change in the rate of glucose appearance and disappearance, substrate oxidation, c-peptide secretion, or endogenous glucose production. The authors suggested that, since insulin secretion and insulin clearance as measured by the c-peptide to insulin molar ratio remained stable, the decrease in insulinemia was indicative of improved peripheral insulin sensitivity [131]. This study corroborated their previous findings in five healthy volunteers who responded with 40% decrease in insulinemia following a CHO load, albeit there was a 6% increase in glycemia [132]. These two studies indicated that fish oil can only partially prevent insulin resistance acutely induced by dexamethasone in humans.

A compelling approach was also introduced by Toktam *et al.* in a cohort of patients with schizophrenia or bipolar disorder treated with second-generation antipsychotics, which are unfavorably related to hyperglycemia and insulin resistance. The study was designed in a randomized double-blind fashion to evaluate the early intervention with omega-3 fatty acids supplementation on insulin resistance, caused by concomitant treatment with olanzapine and a sodium valproate or lithium combination. The study outcome was negative and *n*-3 PUFA did not change HOMA-IR, although there were trends of improvement in fasting insulin; yet no data were generated on postprandial insulin action, which is expected to be impaired with long-term antipsychotic treatment [133]. Of note,

the intervention was of short duration, six weeks, with a very low dose of omega-3 fatty acids, less than 1 g/day, and none of the groups exhibited hyperglycemia with the antipsychotic treatment [82], suggesting that further evaluation is warranted in this group of patients.

5. EPA and DHA Effect on Skeletal Muscle Metabolism

5.1. Skeletal Muscle Lipid Content

Lipotoxicity from ectopic lipid accumulation in skeletal muscle tissue due to conditions of caloric surplus has been purported to contribute to the development of insulin resistance [14,134]. Leading hypotheses, however, do not implicate increased absolute levels of TG, but incomplete lipid β-oxidation and the accumulation of toxic intermediates, including long-chain acylcarnitines (LCACoA) [135], ceramides [136], and diacylglycerols (DAG) [137], which interfere with normal mitochondrial function and insulin signaling [138]. Indeed, in a mouse model of HFD-induced insulin resistance, we showed that muscle triglycerides accumulation was not prevented by fish oil, yet insulin sensitivity improved. This was due to a partial attenuation of LCACoA levels in all muscle fractions of total homogenate, sarcoplasmic and mitochondrial. Also, fish oil attenuated the accumulation of the most abundant ceramide species (palmitic and stearic) [35]. This is consistent with a report of combined treatment with rosiglitazone and *n*-3 PUFA in mice during a HFD, which mitigated the increase in ceramide content, and improved insulin sensitivity [139]. In agreement, Stephens *et al.* demonstrated that acute administration of *n*-3 PUFA in an intravenous lipid emulsion, altering the *n*-6:*n*-3 ratio, did not change total muscle acylcarnitine content; nevertheless, it prevented much of the decline in insulin sensitivity and in pyruvate dehydrogenase complex activity (PDCa), a rate-limiting enzyme in glucose oxidation, which can be allosterically inhibited by *n*-6 PUFA administration [92]. Therefore we conclude that improvements in peripheral insulin sensitivity by *n*-3 PUFA are not necessarily mediated by changes in total lipid content.

5.2. Skeletal Muscle Mitochondrial Function

Mitochondrial dysfunction is intertwined with the insulin resistance phenotype, as these organelles are responsible for lipid oxidation and for sustaining the metabolic demands of skeletal muscle. In this regard, we reported that fish oil increased expression of master transcriptional factors of mitochondrial biogenesis, including PGC1a and nuclear respiratory factor 1 (nrf1) [35]. Others also showed that omega-3 fatty acids are ligands for receptors that activate PGC1a [140], leading to an increased expression of PGC1a, mitochondrial transcription factor A (TFAM), cytochrome c oxidase, and mitochondrial membrane potential [141]. These studies indicate that dietary *n*-3 PUFA can prevent or reverse impairments in muscle mitochondria content or function. However, maximal mitochondrial oxidative capacity and phosphorylation efficiency did not change, pointing towards other mechanisms being associated with insulin signaling. This is consistent with reports in insulin-resistant humans, where oxidative capacity did not change after six months of EPA and DHA supplementation, measured *in vitro* from muscle biopsies and verified *in vivo* with magnetic resonance spectroscopy [76]. In light of these observations, Herbst *et al.* showed that EPA and DHA are incorporated into mitochondrial membranes and exert their effect by increasing mitochondrial sensitivity for ADP, and thus increasing submaximal, but not maximal, oxidative capacity [142]. Nevertheless, in the context of aging, where mitochondrial function is known to be impaired [13], EPA, but not DHA, partially improved mitochondrial oxidative capacity in old mice, without stimulating mitochondrial biogenesis or restoring age-related reductions in mitochondrial abundance. The effects were rather mediated by an improvement in mitochondrial protein quality by reducing deleterious post-translational modifications [32]. These findings would suggest that, depending on the underlying dysfunction (lipid overload, insulin resistance, aging) or severity, there could be differential effects of *n*-3 PUFA on skeletal muscle mitochondria function. In the context of aging, there are no available data, to date, on aged humans to support this assumption. However, we have unpublished data on elderly humans

who were supplemented with 3.9 g/day of EPA/DHA for four months, which demonstrated that EPA/DHA could not reverse the age-related reduction in mitochondrial oxidative capacity, despite favorable effects on muscle protein homeostasis.

6. Conclusions

Despite promising strong outcomes in animal studies, there is no substantial cumulative evidence, to date, that VLC *n*-3 PUFA dietary supplementation can serve as a therapeutic strategy for insulin resistance in humans. Even less promising, are the data in regulating glycemic control in patients with type 2 diabetes. However, there is a strong indication that VLC *n*-3 PUFA may be used as preventive strategies based on their pleiotropic effects and their potential in regulating inflammation and innate immunity at the level of macrophages and the paracrine or endocrine function of cytokines. Although EPA and DHA have been mostly studied together, additional RCTs are needed to delineate their differential and independent effects elicited on muscle, liver, and adipose tissue with regards to insulin action. Further exploration and understanding of these mechanisms could be beneficial for long-term preventive strategies for chronic diseases and for promoting favorable outcomes in the acute clinical setting.

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Postprandial Differences in the Amino Acid and Biogenic Amines Profiles of Impaired Fasting Glucose Individuals after Intake of Highland Barley

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Abstract: The aim of this study was to measure the postprandial changes in amino acid and biogenic amine profiles in individuals with impaired fasting glucose (IFG) and to investigate the changes of postprandial amino acid and biogenic amine profiles after a meal of highland barley (HB). Firstly, 50 IFG and 50 healthy individuals were recruited for the measurement of 2 h postprandial changes of amino acid and biogenic amine profiles after a glucose load. Secondly, IFG individuals received three different loads: Glucose (GL), white rice (WR) and HB. Amino acid and biogenic amine profiles, glucose and insulin were assayed at time zero and 30, 60, 90 and 120 min after the test load. The results showed fasting and postprandial amino acid and biogenic amine profiles were different between the IFG group and the controls. The level of most amino acids and their metabolites decreased after an oral glucose tolerance test, while the postprandial level of γ-aminobutyric acid (GABA) increased significantly in IFG individuals. After three different test loads, the area under the curve for glucose, insulin, lysine and GABA after a HB load decreased significantly compared to GL and WR loads. Furthermore, the postprandial changes in the level of GABA between time zero and 120 min during a HB load were associated positively with 2 h glucose and fasting insulin secretion in the IFG individuals. Thus, the HB load produced low postprandial glucose and insulin responses, which induced changes in amino acid and biogenic amine profiles and improved insulin sensitivity.

Keywords: postprandial status; amino acid and biogenic amine profiles; insulin; highland barley; impaired fasting glucose

1. Introduction

Impaired fasting glucose (IFG) was introduced as a new category of glucose tolerance by the American Diabetes Association and the World Health Organization in order to identify early diabetes [1]. In the USA, the prevalence of IFG was 26.0% in adults [2] and 13.1% in adolescents [3], whereas the prevalence was 0.9%–7.1% in China and Japan [4]. IFG is a state in glucose metabolism intermediate between normal glucose tolerance and type 2 diabetes (T2D) [5]. IFG individuals are characterized primarily by insulin resistance (IR), impaired insulin sensitivity and impaired beta cell function [4–6].

Recent research suggested amino acids might be important in the development of IR with regard to alterations in circulating levels of several amino acids, including aromatic amino acids (AAA) and branched-chain amino acids (BCAA) [7,8]. Baseline levels of AAAs and BCAAs are prognostic for improvement in insulin sensitivity in response to dietary/behavioral intervention [9] and other types of amino acids might be relevant in the development of IR and T2D [10,11]. Most of these studies have been done in the fasting state but it is important to note the body is in the postprandial state for

most of the day. Changes in metabolism in the postprandial state might contribute to alteration of the physiological functions of the body. It is necessary, therefore, to study the potential effect of metabolic changes of amino acid and biogenic amine profiles in the postprandial state, which will be helpful in exploring the relationship between the postprandial amino acid and biogenic amine profiles and IR.

Time-dependent variations in the hormonal and metabolic profiles in the postprandial state are of great importance to human health. An important change in the postprandial state is acute hyperglycemia following a glucose load or a meal, which leads to metabolic changes, including the levels of insulin, fatty acids, and amino acids *etc.* An oral glucose tolerance test (OGTT) or different meals are usually used to investigate these postprandial time-dependent variations. There have been many human studies using an OGTT to investigate the postprandial change of metabolic profiles in diabetic and IR individuals [12,13]. Bondia-Pons *et al.* reported that rye bread and white wheat bread have an effect on postprandial metabolic profiles in healthy individuals [14]. Therefore, it is necessary to investigate systemically the effect of equivalent loads of glucose in the form of highland barley (HB) and white rice (WR) on the postprandial amino acid and biogenic amine profiles in IFG individuals.

The objectives of this study were: (1) to investigate the difference of postprandial amino acid and biogenic amine profiles between IFG and healthy individuals using an OGTT and a targeted metabolomics approach; and (2) to evaluate the effect of a HB load on amino acid and biogenic amine profiles in IFG individuals and to explore the association of these postprandial profiles with IR, thereby opening new perspectives in the study of the postprandial physiological reaction of IFG individuals following glucose ingestion or an HB load.

2. Experimental Section

This study was approved by the Ethics Committee of Harbin Medical University, China. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant. The clinical register number was ChiCTR-TRC-12002630.

All participants were recruited in 2013 from the Hexing and Yixing Districts in Harbin City, Heilongjiang Province in northern China via posters in the Districts. The glycemic state was classified according to the American Diabetes Association criteria [15] after a 75 g OGTT. Participants with fasting plasma glucose (FPG) levels between 6.1 and 6.9 mmoL/L and a 2 h post challenge glucose (2h-PG) level of <7.8 mmoL/L were identified as IFG. The exclusion criteria were: any cardiovascular complication or inflammatory disease, and any medications such as antioxidants and lipid-lowering or glucose-lowering drugs. The design of this study is illustrated in Supplementary Figure S1.

Data for age, weight, height, alcohol use, cigarette smoking, menstrual status, as well as physical activity at work and at leisure was obtained from questionnaires. Dietary intakes were estimated with a validated semi-quantitative food frequency questionnaire.

2.1. Study 1: Amino Acid and Biogenic Amine Profiles Change in IFG Individuals during 120 min OGTT

IFG (50) and control participants (50) were recruited according to FPG and 2h-PG levels. There was no significant difference ($p > 0.05$) between the two groups with regard to daily physical activity level (data not shown), age, gender, cigarette smoking, alcohol consumption or dietary intake (Table 1). Body mass index (BMI), systolic blood pressure, diastolic blood pressure, levels of triglycerides (TGs) and total cholesterol (TC), FPG, 2h-PG and hemoglobin A1c (HbA_{1c}%) were significantly different ($p < 0.05$) between IFG individuals and controls (Table 1).

After 12 h fasting, blood samples were collected and participants were challenged with the equivalent of 75 g anhydrous glucose dissolved in 250 mL water. No food or drink was consumed during the test. After 2 h, post-challenge blood samples were collected, separated by centrifugation ($3000\times g$ for 15 min) at room temperature and the supernatant was stored at –80 °C.

Table 1. Demographic and Clinical Chemistry Characteristics of Human Subjects.

Parameter	Control (n = 50)	IFG (n = 50)	p value
Sex (femeal/male)	16/14	15/15	0.82
Age (years)	44.86 ± 9.48	45.86 ± 10.55	0.16
Smoker/non-smoker	12/18	13/17	0.67
Protein (g/day)	80.34 ± 12.23	81.21 ± 11.13	0.56
Fat (g/day)	71.45 ± 21.27	72.10 ± 22.09	0.39
Carbohydrate (g/day)	324.01 ± 108.17	325.07 ± 109.21	0.68
BMI (kg/m^2)	22.24 ± 1.66	24.61 ± 5.30	<0.001
TC (mmmoL/L)	4.16 ± 0.51	4.59 ± 1.24	<0.001
TG (mmmoL/L)	0.90 ± 0.32	1.59 ± 0.53	<0.001
Fasting glucose (mmmoL/L)	4.03 ± 0.47	5.69 ± 0.42	<0.001
2 h-glucose (mmmoL/L)	4.67 ± 0.99	6.13 ± 1.14	<0.001
SBP (mmHg)	112.70 ± 6.43	136.62 ± 15.40	<0.001
DBP (mmHg)	75.07 ± 6.02	80.16 ± 9.44	<0.001
HAc1 (%)	5.2 ± 0.2	6.2 ± 0.3	0.008
Fasting insulin (mU/L)	6.07 ± 2.12	7.19 ± 2.42	0.02
2 h-insulin (mU/L)	8.55 ± 2.08	23.45 ± 2.5	<0.001
HOMR-IR	1.09 ± 0.46	1.90 ± 0.46	<0.001

SBP: Systolic blood pressure; DBP: Diastolic blood pressure; TG: Triglycerides; TC: Total cholesterol. FBG: Fasting plasma glucose; 2 h-PG: 2 h Postprandial plasma glucose.

2.2. Study 2: Postprandial Amino Acid and Biogenic Amine Profiles after Three Test Loads in IFG Individuals

IFG individuals in study 1 were given glucose (GL), WR and HB test loads. The WR load was white rice (Qizheng Company, Lanzhou, China) and the HB load was highland barley (Qizheng Company, Lanzhou, China). Glucose was purchased from the pharmacy (Harbin Medical University, Harbin, China). WR and HB loads were boiled before they were served. The amounts of WR load (89.7 g) and HB load (100.2 g) were calculated to provide the total energy available from 75 g glucose. Details of the test loads are given in Supplementary Table S1. Participants were given a test load at 9 A.M. (breakfast) and were required to consume it within 20 min. Blood samples were collected at time zero (9 A.M.) and at 30, 60, 90 and 120 min later, centrifuged immediately at 3000× g for 15 min at room temperature and the supernatant was stored at –80 °C.

Each test day was separated by a washout period of seven days. All participants were asked to avoid heavy exercise and intake of alcohol 24 h before each test day. The day before each test day, participants were provided with a standardized meal and snack, which contained 15% (w/v) protein, 50% (w/v) carbohydrate and 35% (w/v) fat. The subjects were instructed to eat and drink the same prescribed foods at 8 A.M. on each test day.

2.3. Biochemical Measurements

Serum glucose, TC, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c) and TGs were measured with kits purchased from Biosino Biotechnology (Beijing, China), standard enzymatic colorimetric techniques and with an auto-analyzer (MOL-300, Beijing, China). Hemoglobin A1c (HbA1c) was measured by a hemoglobin A1c analyzer on a BX5DS5Menarini-ArkrayKDKHA8140 (Arkray, Kyoto, Japan). Serum insulin was measured with an auto-analyzer using commercial kits (Centaur, Bayer Corporation, Bayer Leverkusen, Germany).

2.4. Serum Preparation

Serum amino acids and biogenic amines were prepared as described [16]. Briefly, each serum sample (50 μL) was used for metabolite extraction before UPLC-TQ-MS analysis. The metabolite extraction procedure was carried out after adding 250 μL of acetonitrile/methanol/formic acid (74.9:24.9:0.2 by vol.) containing two additional stable isotope-labeled internal standards for valine-d8 and phenylalanine-d8 in serum. After vortex mixing for 1 min, the mixture was kept at room

temperature for 10 min and then centrifuged at 14,000× *g* for 10 min at 4 °C. The solution was filtered through a syringe filter (0.22 μm pore size) then placed into a sampling vial for UPLC-TQ-MS analysis.

2.5. UPLC-TQ-MS Analysis

UPLC-TQ-MS analysis was done with a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) coupled to a Waters Xevo TQD mass spectrometer (Waters Corporation, Manchester, UK). A portion (2 μL) of the sample solution was injected into an ACQUITY UPLC™ HILIC column (100 mm × 2.1 mm *i.d.*, 1.7 μm film thickness; Waters Corporation, Milford, MA, USA). The flow rate of the mobile phase was 300 μL/min. Analytes were recovered from the column by gradient elution using solution A (10 mM aqueous ammonium formate, 0.1% (*v*/*v*) formic acid) and solution B (0.1% (*v*/*v*) formic acid in acetonitrile). The optimized conditions for the UPLC separation and ESI-TQ-MS detection are given in Supplementary Table S2.

MS analyses used electrospray ionization (ESI) and multiple reaction monitoring scans in the positive ion mode. Cone voltage and collision energies for 30 ms were optimized for each transition, the ion spray voltage was 3.2 kV and the source temperature was 150 °C. Internal standard peak areas were monitored for quality control and individual samples with peak areas differing from the group mean by more than two standard deviations were reanalyzed. MarkerLynx Application Manager software (version 4.1; Waters Corporation, Milford, MA, USA) was used for automated peak integration and metabolite peaks were reviewed manually for quality of integration and compared against a known standard to confirm identity.

2.6. Statistical Analysis

Values are presented as mean ± SD. Statistical analyses were done with SPSS 13.0 software (SPSS, Chicago, IL, USA). $p \leq 0.05$ was considered statistically significant. The area under the curve (AUC) was calculated using the trapezoidal rule to quantify overall response to different loads, which reflected both the amount and duration of the response. Significance was determined by the two-tailed Student's *t* test, analysis of covariance (ANCOVA) and repeated-measures ANOVA followed by the Tukey *post hoc* test.

Amino acid and biogenic amine variables between IFG individuals and controls were analyzed by ANCOVA adjusting for potential covariates (BMI, TG, TC, 2 h-PG, blood pressure and insulin level). The time course of glucose, insulin and postprandial amino acid and biogenic amine responses were analyzed by repeated-measures ANOVA followed by a Tukey *post hoc* test. Differences in the postprandial response between time and different loads were assessed via time × test load interaction tests. The correlation of postprandial changes in amino acids, fasting glucose and insulin, 2 h glucose and insulin, and insulin secretory indices during three test loads was assessed using Pearson's correlation test.

3. Results

3.1. Study 1: The Amino Acid and Biogenic Amine Profiles Change in the Control and IFG Participants during 120 min OGTT

In the fasting state, 30 amino acids and biogenic amines were detected in all participants (Supplementary Table S3). The levels of 14 metabolites changed significantly in the IFG group compared to the controls. Compared with the control, ten metabolites (leucine, valine, isoleucine, phenylalanine, alanine, creatinine, glutamic acid, aminobutyric acid, cotinine and L-α-glycerophosphorylcholine, increased significantly ($p < 0.05$) and the levels of four metabolites (methionine, γ-aminobutyric acid (GABA), asparagine and allantoin) decreased.

In both control and IFG participants, 18 metabolites were unaltered in the postprandial state compared to baseline levels (Figure 1). Levels of 12 metabolites in IFG individuals and 11 metabolites in controls were altered significantly ($p < 0.05$) according to the OGTT (Figure 1C,D). In the controls,

ten metabolites decreased and only creatine increased compared to the fasting state (Figure 1C). Ten metabolites were decreased at 120 min in IFG individuals compared to the fasting state, whereas GABA and cysteine were increased (Figure 1D). GABA was the important metabolite with different dynamic change during OGTT between the IFG and control groups. Postprandial GABA was increased (fold change 1.39 ± 0.45) at 120 min in IFG participants and was decreased significantly in the controls (fold change 0.43 ± 0.18).

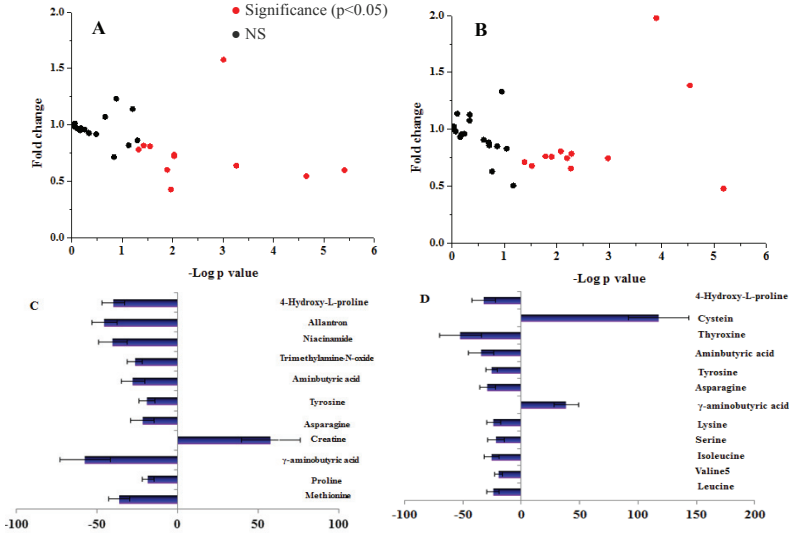

Figure 1. Fold change and significance of metabolite change during an oral glucose challenge in the control (**A**) and the IFG (**B**) groups, and significant percent change of metabolites from fasting to 2-h samples during an OGTT in control (**C**) and the IFG (**D**) groups. Dots represent the 30 metabolites detected in serum. Significant ($p < 0.05$) changes are colored red. Fold changes and percent changes for the metabolites (X) detected by UPLC-TQ-MS in three loads were calculated as follows: $X_{\text{Fold change}} = (X_{\text{Concentration at different time (30, 60, 90, 120 min)}} / X_{\text{Concentration at baseline}})$; $X_{\text{Percent change}} = (X_{\text{Concentration at different time (30, 60, 90, 120 min)}} - X_{\text{Concentration at baseline}}) / (X_{\text{Concentration at baseline}})$.

3.2. Study 2: The Postprandial Amino Acid and Biogenic Amine Profiles after Three Test Loads in the IFG Group

3.2.1. Glucose and Insulin Profiles after Three Test Loads

The levels of serum glucose and insulin following different test loads showed a postprandial increase followed by a decrease (Figure 2A,C). There were significant effects of test load, time and time × test load interaction on glucose (test load $p < 0.001$; time $p < 0.001$; interaction $p < 0.002$) and insulin (test load $p < 0.001$; time $p < 0.001$; interaction $p < 0.001$). At 0–120 min the AUC for serum glucose and insulin was significantly smaller for the HB load compared to WR and GL loads (Figure 2B,D).

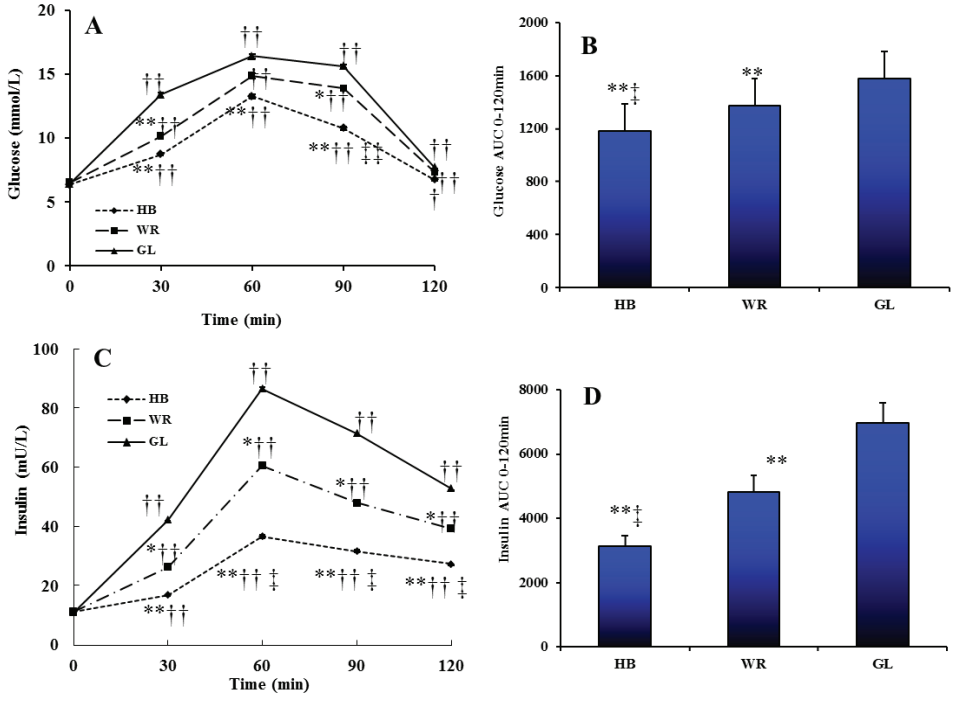

Figure 2. The changes in serum glucose level (**A**) and insulin level (**C**) during tree test loads. The AUC of glucose (**B**) and insulin (**D**) between 0 and 120 min of different test loads. Triangle, glucose load (GL); box, WR load (WR); diamond, HB load (HB). Repeated-measures ANOVA showed significant main effects of test load, time or a time × test load interaction on glucose (test load, $p < 0.001$; time, $p < 0.001$; interaction, $p = 0.002$) and insulin (test load, $p < 0.001$; time, $p < 0.001$; interaction, $p < 0.001$). * $p < 0.05$, ** $p < 0.01$, HB or WR *vs.* GL at the same time point using repeated measures ANOVA analysis with a Tukey *post hoc* test. ‡ $p < 0.05$, ‡‡ $p < 0.01$, HB *vs.* WR at the same time point of the ingesting WR using repeated measures ANOVA analysis with LSD *post hoc* test. † $p < 0.05$, †† $p < 0.01$, compared with the baseline in the same treated group using multiple comparisons analysis with LSD *post hoc* test.

3.2.2. The Postprandial Amino Acid and Biogenic Amine Profiles after Three Test Loads

The fold change and significance of metabolite changes during three test loads in IFG participants are shown in Figure 3 and Supplementary Figures S2–S4. The number of significant metabolites increased from time zero–120 min for GL and WR loads, whereas the number of significant metabolites was not altered from time zero–90 min and decreased at 120 min during the HB load. With regard to the change of metabolites at 120 min, the levels of 15 metabolites after a GL load (Supplementary Figure S2) and 20 metabolites after a WR load (Supplementary Figure S3) were changed significantly and only four metabolites decreased significantly after a HB load (Supplementary Figure S4). The changed levels of GABA were different among the three test loads. The postprandial level of GABA was higher after GL and WR loads but was not altered significantly at 30, 60 or 90 min and was decreased significantly (−15.5%) at 120 min for a HB load. The mean concentrations of GABA after a HB load were significantly lower ($p < 0.05$) at 30, 60, 90 and 120 min compared to GL and WR loads (Figure 4F). For the postprandial change in GABA after the three test loads, there were significant differences ($p < 0.001$) in the time, test load and the interaction of time × test load (Figure 4). The 0–120 min AUC for GABA after an HB load was significantly lower ($p < 0.05$) compared to GL and WR loads (Supplementary Figure S5F).

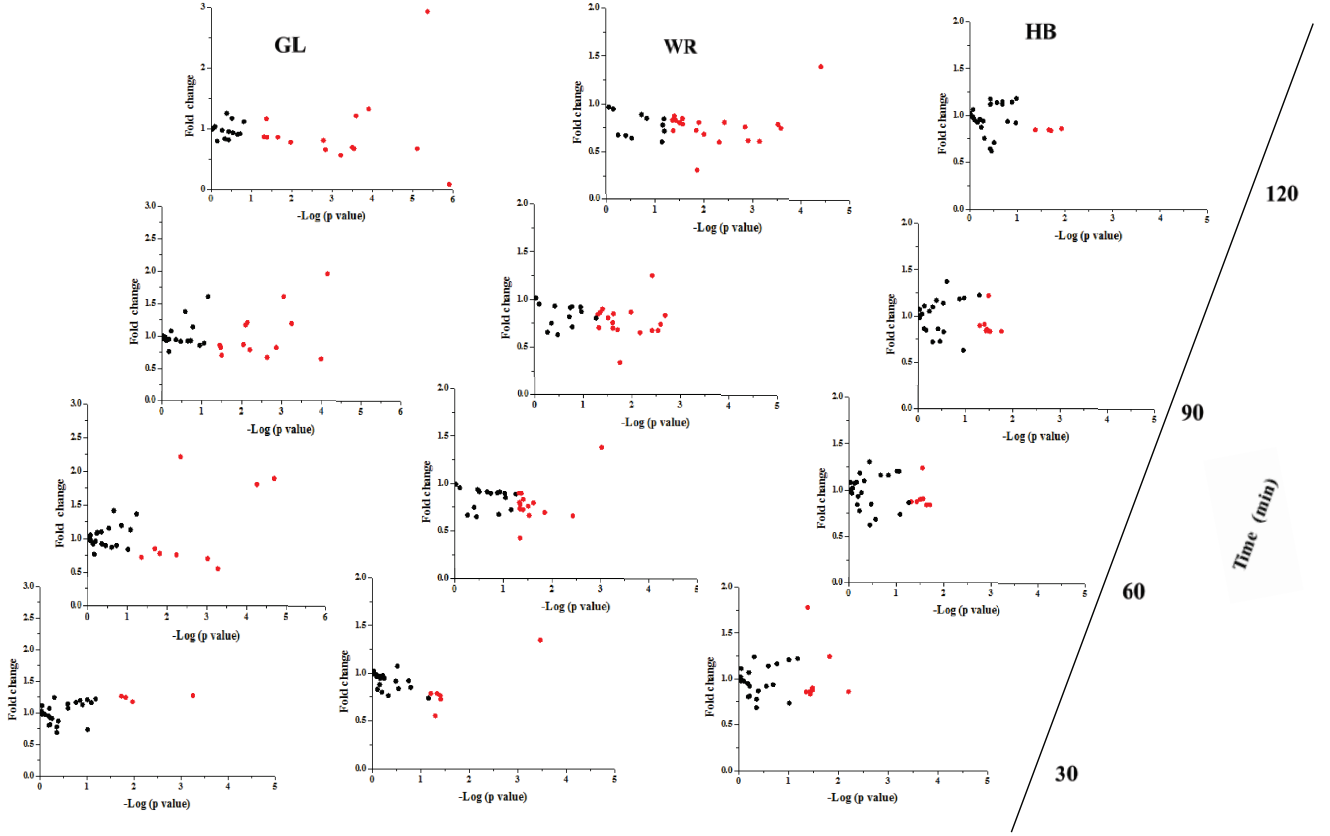

Figure 3. Fold change and significance of metabolite change in the IFG group during different test loads. Fold changes and percent changes for the metabolites (X) detected by UPLC-TQ-MS in three loads were calculated as follows: $X_{\text{Fold change}} = (X_{\text{Concentration at different time (30, 60, 90, 120 min)}}/X_{\text{Concentration at baseline}})$; $X_{\text{Percent change}} = (X_{\text{Concentration at different time (30, 60, 90, 120 min)}} - X_{\text{Concentration at baseline}})/(X_{\text{Concentration at baseline}})$.

3.2.3. Correlation between Postprandial Metabolites, Glucose and Insulin Following Different Loads

Postprandial changes in the concentrations of leucine, histidine and lysine during the three test loads were correlated with 2h-insulin (Table 2), whereas the postprandial change of alanine was correlated negatively ($p < 0.05$). GABA was associated positively with 2 h-glucose during three test loads with 2 h-glucose and fasting insulin in the GL and WR loads, while GABA was related negatively to 2 h-glucose in the HB load. The postprandial dynamic change in six significant metabolites correlated with glucose and insulin during the three test loads is shown in Figure 4 and Supplementary Figure S5. For the postprandial change in six significant metabolites, there was a significant difference ($p < 0.05$) in the interaction of time × test load (Figure 4). AUC for leucine, valine and histidine for the HB load was significantly greater ($p < 0.05$) compared to the GL load and AUC for lysine and GABA was significantly smaller ($p < 0.05$) compared to the GL load.

Table 2. Associations between glucose, insulin and insulin sensitivity index and percent change of metabolites from fasting to 2-h sample response to three test loads.

Metabolites	**GL**			
	Fasting Glucose	**2 h-glucose**	**Fasting Insulin**	**2 h-Insulin**
Leucine				−0.647 (0.024)
Valine				
Histidine				−0.604 (0.029)
Alanine		−0.600 (0.048)		
Lysine				−0.587 (0.034)
γ-aminobutyric acid		0.621 (0.023)	0.551 (0.040)	
	HB			
	Fasting glucose	2 h-glucose	Fasting insulin	2 h-insulin
Leucine				−0.774 (0.023)
Valine				
Histidine				−0.637 (0.045)
Alanine		−0.607 (0.041)		
Lysine				−0.548 (0.042)
γ-aminobutyric acid		0.614 (0.038)	0.514 (0.044)	
	WR			
	Fasting glucose	2 h-glucose	Fasting insulin	2 h-insulin
Leucine				−0.627 (0.038)
Valine				
Histidine		0.691 (0.018)		−0.621 (0.029)
Alanine		−0.761 (0.006)		
Lysine				−0.568 (0.042)
γ-aminobutyric acid		−0.572 (0.041)		

Data was analyzed by Pearson correlation. Data for metabolites, glucose, insulin and insulin sensitivity index associations, which did not achieve statistical significance are not included. Data are presented as standardized correlation-coefficients (*p*-values). Standardized correlation-coefficients were computed from standard deviations with *p*-values < 0.05.

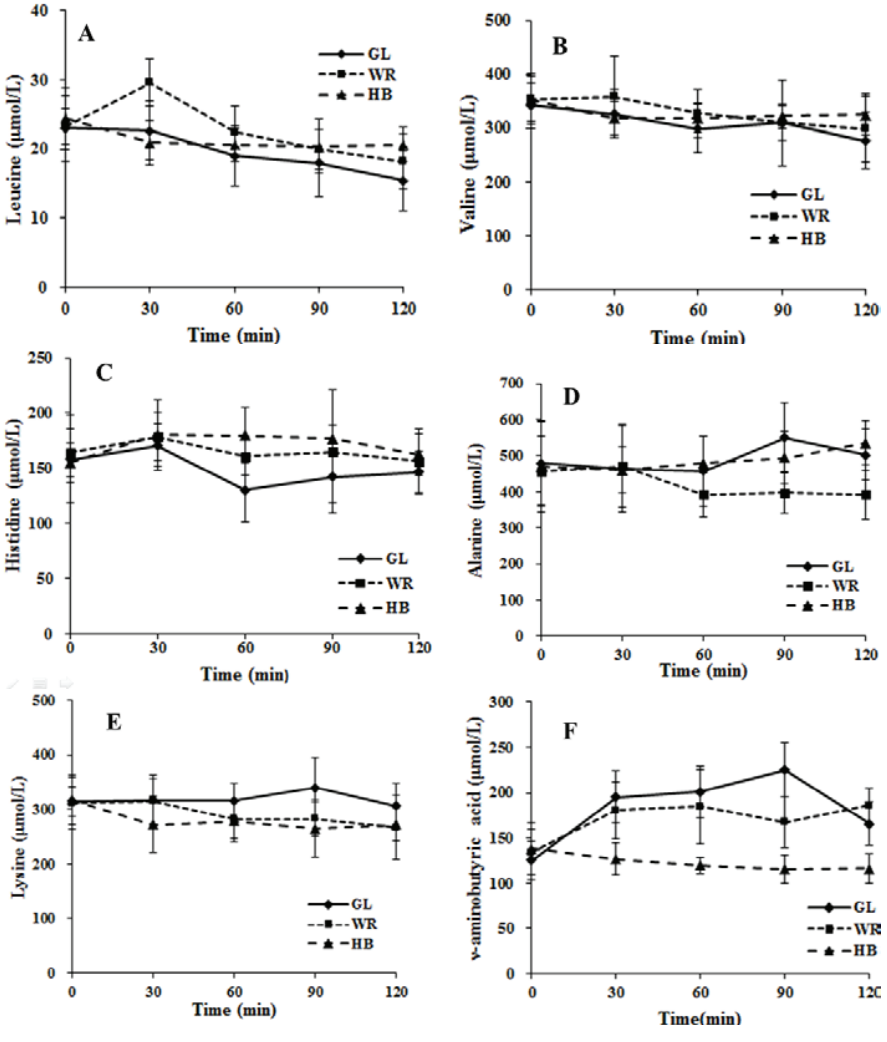

Figure 4. The changes in serum leucine (**A**), valine (**B**), histidine (**C**), alanine (**D**), lysine (**E**) and γ-aminobutyric acid (**F**) during three test loads. Diamond, glucose load (GL); box, WR load (WR); Triangle, HB load (HB). Repeated-measures ANOVA showed significant main effects of test load, time or a time × test load interaction on leucine (test load, $p = 0.041$; time, $p < 0.001$; interaction, $p = 0.005$), valine (test load, $p = 0.199$; time, $p = 0.001$; interaction, $p = 0.013$), histidine (test load, $p = 0.008$; time, $p < 0.001$; interaction, $p < 0.001$), alanine (test load, $p = 0.009$; time, $p = 0.297$; interaction, $p = 0.005$), lysine (test load, $p = 0.016$; time, $p = 0.013$; interaction, $p = 0.028$), γ-aminobutyric acid (test load, $p < 0.001$; time, $p < 0.001$; interaction, $p < 0.001$).

4. Discussion

In this study, there were different fasting amino acid and biogenic amine profiles and postprandial response of amino acid and biogenic amine profiles between control and IFG participants. Recently, the idea that BCAAs and several related amino acids are linearly related to the homeostasis model assessment of insulin resistance has been supported by the results of some studies [8,17]. Other studies

demonstrated elevated levels of BCAA were associated strongly with the future risk of diabetes and valine might be a biomarker for the identification of pre-diabetes such as IFG [8,18]. Our results suggested there were higher levels of AAAs and BCAAs in the IFG group compared to the controls, suggesting a high level of BCAAs in a clinical study might imply the risk of IFG or diabetes. Moreover, our study confirmed there were different metabolic changes in postprandial amino acid and biogenic amine profiles in IFG individuals during three test loads. The postprandial changes of glucose and insulin were lower after a HB load compared to GL and WR loads. Especially, there was a smaller AUC for GABA after a HB load compared to GL and WR loads. These results suggested a HB load lowered the response of insulin and further altered postprandial amino acid and biogenic amine profiles.

With regard to the regulation of the postprandial response of insulin production to a HB load, it is reported that oats and barley can decrease the insulin response because they contain β-glucan [19]. Our results demonstrated the HB load might decrease the postprandial response of insulin. The high β-glucan content (6.42%, *w*/*w*) of the HB load might be the main reason for reduction of the postprandial insulin response in this study. A potential mechanism might be related to changes in gut hormones, including GLP-1. Some studies demonstrated plasma insulin responses were associated closely with GLP-1 [20,21] and the secretion of GLP-1 is known to be influenced by diet [22]. A recent study, as well as our unpublished observations, showed there was a low level of GLP-1 during an HB load. Therefore, a low level of postprandial insulin might be attributed, at least in part, to a decrease of GLP-1 associated with consumption of a carbohydrate load containing a high content of β-glucan.

Insulin is associated closely with amino acid metabolism in the postprandial state [12]. Earlier studies showed acute hyperglycemia after a glucose load resulted in change of the amino acids profiles in healthy adults, obese individuals and impaired glucose tolerance individuals because the metabolism in the body was regulated by the postprandial level of insulin [12,23]. In agreement with these studies, there was postprandial change of amino acid and biogenic amine profiles in the IFG participants and controls in the present study. There were different metabolic alterations after the three test loads in IFG participants and the number of metabolites changed by a HB load was lower compared to GL and WR loads. The HB load contained a high level (6.42%, *w*/*w*) of β-glucan, which has been reported to prolong the postprandial insulinemic response, leading to a decreased level of postprandial insulin [24]. Therefore, the low level of insulin during a HB load in this study probably inhibited the postprandial change of some metabolites. Moreover, our data demonstrated that the change in postprandial BCAAs (leucine and valine) was correlated with insulin. BCAAs, essential amino acids for humans, have central roles in protein metabolism [25], improving glucose metabolism [26] and regulating leptin secretion during food intake [27]. Some reports have shown a gradual decrease of BCAAs during an OGTT in healthy and the impaired glucose tolerance subjects [12,23] in accord with this study. Furthermore, there were smaller decreases of these amino acids associated with an HB load compared to GL and WR loads. BCAAs are particularly sensitive to insulin action and their metabolism has been observed to be altered profoundly in insulin-resistant states. Recently, Newgard *et al.* [8] observed associations between BCAAs and insulin resistance and Tai *et al.* [17] reported insulin resistance was associated with leucine/isoleucine. DeFronzo *et al.* suggested first-phase insulin secretion is important for the inhibition of endogenous glucose production during an OGTT or a meal [28], so the less severe decrease of BCAAs during a HB load might be due to already decreased levels of postprandial insulin and the increase in early-phase insulin secretion in HB load would be expected to increase hepatic glucose production and suppress the excessive rise in plasma glucose. In short, our results suggested these postprandial changes in several amino acids, especially BCAAs, could shed new light on postprandial metabolic dysregulation in IFG individuals and HB might be helpful for controlling such dysregulation.

Another important finding of this study was the less pronounced increase of GABA during an OGTT in IFG individuals, and that the change in GABA was different among the three test loads (Figure 4). There were higher postprandial levels of GABA after GL and WR loads, whereas postprandial levels of GABA decreased significantly (−15.5%) at 120 min for a HB load. In the pancreas,

GABA is produced primarily by insulin-secreting beta cells [29] and the release of GABA from these cells appears to be regulated by glucose [30]. Therefore, different levels of glucose in the three test loads might contribute to the different postprandial changes in GABA levels. Moreover, GABA had antioxidative effects [31] and acute hyperglycemia after a meal or glucose load increases oxidative stress [32]. Thus, increased GABA with GL and WR loads might be a stress response to ameliorate or prevent the high postprandial oxidative stress in IFG individuals. In summary, different postprandial GABA among the three test loads indicated GABA was regulated by diet.

This study has several limitations: Firstly, the duration of the postprandial investigation was short (only 2 h), thus, it is relevant only to the short-term effects following a carbohydrate load. Secondly, the effect of different proteins in the WR and HB loads on the postprandial amino acid and biogenic amine profiles was not considered. Finally, the number of participants was small and care must be exercised in the extrapolation of our findings to larger populations. Therefore, further studies are needed in this area.

5. Conclusions

In conclusion, our study found the amino acid and biogenic amine profiles were different between IFG individuals and controls at baseline and after an OGTT. This study showed also that a HB load was associated with a low postprandial glucose and insulin response, which resulted in changes of the amino acid and biogenic amine profiles. The results of this study offer new insights into the complex physiological regulation of metabolism in IFG individuals during the consumption of different sources of dietary carbohydrate.

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

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Section 3:
Diet and Genetic Background

nutrients

MDPI

Article

Homocysteine Metabolism Gene Polymorphisms (MTHFR C677T, MTHFR A1298C, MTR A2756G and MTRR A66G) Jointly Elevate the Risk of Folate Deficiency

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Abstract: Folate deficiency is strongly associated with cardiovascular disease. We aimed to explore the joint effect of the methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C, methionine synthase (MTR) A2756G, and methionine synthase reductase (MTRR) A66G polymorphisms on folate deficiency in a Chinese hypertensive population. A total of 480 subjects aged 28–75 were enrolled in this study from September 2005–December 2005 from six hospitals in different Chinese regions. Known genotypes were detected by PCR-RFLP methods and serum folate was measured by chemiluminescence immunoassay. Our results showed that *MTHFR* 677TT and *MTR* 2756AG + GG were independently associated with a higher risk of folate deficiency (TT *vs.* CC + CT, $p < 0.001$ and AG + GG *vs.* AA $p = 0.030$, respectively). However, the *MTHFR* A1298C mutation may confer protection by elevating the serum folate level ($p = 0.025$). Furthermore, patients carrying two or more risk genotypes showed higher odds of folate deficiency than null risk genotype carriers, especially those carrying four risk genotypes. These findings were verified by generalized multifactor dimensionality reduction ($p = 0.0107$) and a cumulative effects model ($p = 0.001$). The results of this study have shown that interactions among homocysteine metabolism gene polymorphisms lead to dramatic elevations in the folate deficiency risk.

Keywords: *MTHFR* C677T; *MTHFR* A1298C; *MTR* A2756G; *MTRR* A66G; folate deficiency

1. Introduction

Recently, a large-scale randomized clinical trial confirmed the benefits of folate therapy on the risk of first stroke [1]. A previous meta-analysis has shown that the effect of the *MTHFR* C677T variant on the homocysteine concentration is modified by folate status [2]. Hyperhomocysteinemia and the *MTHFR* 677TT genotype are considered risk factors for cardiovascular diseases (CVDs) [2–5]. The associations between homocysteine metabolism gene polymorphisms and homocysteine, folate, and other B vitamins have been widely studied [6]. Some mutations may result in an elevation in the plasma homocysteine concentration and a reduction in the folate concentration [6–11], thereby exacerbating the risks of several complicated diseases [6,12]. Other studies have demonstrated that

high-dose folate intervention therapy [13] or dietary folate supplementation [8] may increase the serum folate level and simultaneously reduce the prevalence of hyperhomocysteinemia and hypertension.

Folate is a crucial vitamin in homocysteine metabolism. Serum folate enters into tissue cells via folate receptors, and then dihydrofolate reductase (DHFR) converts it into tetrahydrofolate. Next, tetrahydrofolate is transformed into 5, 10-methylenetetrahydrofolate, with vitamin B_6 as a cofactor [14]. Then, methylenetetrahydrofolate reductase (MTHFR) converts 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, providing a methyl group for conversion of homocysteine into methionine in a reaction catalyzed by methionine synthase (MTR) [15,16]. MTR requires vitamin B_{12} (cobalamin) as a coenzyme. Over time, the cobalamin (I) cofactor of MTR is oxidized to form cobalamin (II), leading to inactivation of MTR. Thus, methionine synthase reductase (MTRR) is required for reversion of oxidized cobalamin (II) to CH_3-cobalamin (III) to maintain the activity of MTR [17].

Some common polymorphisms (*MTHFR* C677T, rs1801133; *MTHFR* A1298C, rs1801131; *MTR* A2756G, rs1805087; and *MTRR* A66G, rs1801394) may influence the serum folate level [6,7,10,18]. Numerous studies have demonstrated that the *MTHFR* C677T mutation significantly lowers the serum folate level [7,10,11,19], whereas a recent study has reported no such correlation [20]. The associations of *MTHFR* A1298C and *MTR* A2767G with the folate level remain controversial [10,18]. In addition, the *MTRR* A66G polymorphism itself may not affect the plasma folate level [7]. Further, these mutations may synergistically promote folate deficiency [21,22]. A low folate level may increase the risk of hyperhomocysteinemia, as has been demonstrated in 77% of hypertensive patients in a previous study [23]. In many developed countries (USA, Canada, UK, France, and other western countries), folic acid fortification has been fully implemented. This measure has been reported to reduce the risk of complex diseases [2,6,8]. However, no folate fortification policy has been established in China, India, Pakistan or other Asian countries and, thus, folate deficiency is more common in Asian populations than in European and American populations [9–11].

The aims of our study were to investigate the associations between homocysteine metabolism gene polymorphisms (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G) and the serum folate level, as well as to explore the independent and interactive effects of the risk genotypes on the incidence of folate deficiency in the Chinese hypertensive population.

2. Experimental Section

2.1. Participants and Procedures

This study was conducted using data collected in a previous study [24]. This was a multicenter, randomized, double-blind controlled trial in hypertensive Chinese adults (clinicaltrials.gov; identifier: NCT00520247). Details regarding "Study subjects", "Randomization and double blinding", "Data collection procedures", and "Laboratory tests" have been previously described [24]. In total, 480 patients with mild or moderate hypertension were recruited from six hospitals in different Chinese regions (Ha'rbin, Shanghai, Shenyang, Beijing, Xi'an, and Nanjing) from September to December 2005. All six hospitals have been certified as clinical pharmacology centers by the State Food and Drug Administration of China. Demographic and clinical information and serum folate and homocysteine concentrations were obtained at baseline. This study was approved by the Ethics Committee of Peking University First Hospital, Beijing, China. The purpose and procedures of the study were carefully explained to all participants, and written informed consent was obtained from each participant.

2.2. DNA Extraction and Genotyping

All participants were requested to provide 2 mL peripheral whole blood, which was collected in ethylenediaminetetraacetic acid (EDTA) and stored at −20 °C. DNA was extracted by conventional methods. The TaqMan probe technique was used for detecting polymorphisms in Hcy pathway genes at our central laboratory. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was applied to detect the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and

MTRR A66G genotypes. Each genotyping reaction mixture contained 4 ng dried DNA, 0.08 mL 40 assay locus-specific probe, and 2.0 mL TaqMan universal polymerase chain reaction (PCR) master mix in a final volume of 4 mL, with addition of 1.92 mL sterile water. The main parameters for PCR-RFLP of the four single nucleotide polymorphisms (SNPs) are shown in Table 1. The amplified PCR products were separated on a 3% agarose gel. To ensure the accuracy of genotyping, genotyping calls were observed by two independent researchers. The genotyping call rate for assessments of all genetic variants was ≥98% in this study.

Table 1. Primer sequences and reaction conditions for PCR-RFLP of gene polymorphisms.

Gene	Primer sequence [1]	T [2] and Cycles	Product Size	Restriction Enzyme
MTHFR C677T	F: 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ R: 5′-AGGACGGTGCGGTGAGAGTG-3′	58 °C, 35	198bp	*Hinf* I
MTHFR A1298C	F: 5′-CTTTGGGGAGCTGAAGGACTACTAC-3′ R: 5′-CACTTTGTGACCATTCCGGTTTG-3′	52 °C, 38	163bp	*Mbo* II
MTR A2756G	F: 5′-GAACTAGAAGACAGAAATTCTCTA-3′ R: 5′-CATGGAAGAATATCAAGATATTAGA-3′	53 °C, 36	189bp	*Hae* III
MTRR A66G	F: 5′-GCAAAGGCCATCGCAGAAGACAT-3′ R: 5′-GTGAAGATCTGCAGAAAATCCATGTA-3′	60 °C, 35	151bp	*Nsp* I

[1] F: forward primer; R: reverse primer. [2] T: annealing temperature.

2.3. Statistical Analysis

Statistical analyses were conducting using IBM SPSS software package (version 19.0 for windows; IBM, Inc., Armonk, NY, USA). The results for the categorical variables (*i.e.*, the clinical centers and genotypes) are presented as numbers and percentages of cases. The continuous variables (*i.e.*, age, height, weight, body mass index (BMI), systolic blood pressure (SBP), and diastolic blood pressure (DBP)) are presented as the mean ± standard deviation. The means for the continuous variables in the two groups were compared using t tests, and the prevalences of the categorical variables were compared using χ^2 tests. Since the serum folate and homocysteine levels were not normally distributed, the geometric means and quartiles were displayed and were analyzed with the Mann-Whitney U test. Hardy-Weinberg equilibrium was also assessed for the genotypic frequencies of the different genes with the χ^2 test.

The unitary linear regression model was used to assess the associations of the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G gene polymorphisms with the logarithmic transformed folate level. Unconditional logistic regression (ULR) was performed to estimate the independent and joint effects of the genotypes on folate deficiency, defined as a serum folate level of less than 10 nmol/L [25]. The trend test with the general linear model (GLM) was used to verify the above results. A two-sided p value of <0.05 was considered significant.

Generalized multifactor dimensionality reduction (GMDR, version 0.9, obtained from http://www.ssg.uab.edu/gmdr/) was applied to analyze high-order gene-gene interactions. Some parameters, such as training balance accuracy, testing balance accuracy, p value, and cross-validation consistency (CVC), were obtained. The model with the maximum CVC score, the best prediction accuracy, and a p value of 0.05 or lower was considered the best model. All analyses were adjusted for potential confounding factors, including sex, age, clinical center, height, and weight.

3. Results

A total of 480 patients were recruited for this study. After exclusion of 12 subjects who lacked data on *MTHFR* A1298C (five patients), *MTR* A2756G (six patients) or folate (one patient), a total of 468 subjects were included in our final analysis. Four polymorphisms (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G and *MTRR* A66G) in this population showed no deviation in genotype distribution from expected Hardy-Weinberg equilibrium (p values of 0.711, 0.380, 0.862 and 0.393, respectively). Since

only four subjects had the 2756GG genotype, the 2756AG and 2756GG genotypes were combined for the following statistical analyses.

3.1. Demographic and Clinical Characteristics

There were no significant differences in age, BMI, SBP or the frequencies of the four gene polymorphisms between the males and females (Table 2). However, the females had a higher serum folate level ($p < 0.001$), lower serum homocysteine level ($p < 0.001$), and lower DBP ($p < 0.001$) compared with the males.

Table 2. Clinical and epidemiologic characteristics of the population grouped by sex.

Characteristic	Sex		*p*
	Females (*n* = 268)	Males (*n* = 200)	
Age, year	56.7 ± 9.4	56.7 ± 10.7	0.990
Height, cm	157.6 ± 5.3	169.6 ± 5.9	<0.001
Weight, kg	64.4 ± 9.5	73.3 ± 10.6	<0.001
Body mass index, kg/m^2	25.9 ± 3.6	25.4 ± 3.1	0.115
Systolic blood pressure, mm Hg	154.6 ± 12.0	153.8 ± 11.5	0.465
Diastolic blood pressure, mm Hg	92.0 ± 8.2	95.0 ± 8.7	<0.001
Folate, nmol/L	13.62 (10.48–16.92)	11.50 (8.90–13.40)	<0.001
Homocysteine, μmol/L	11.34 (9.12–14.23)	15.96 (11.50–19.18)	<0.001
	Clinical center		
Ha'rbin	35 (13.1)	24 (12.0)	<0.001
Shanghai	48 (17.9)	13 (6.5)	
Shenyang	44 (16.4)	34 (17.0)	
Beijing	67 (25.0)	47 (23.5)	
Xi'an	30 (11.2)	47 (23.5)	
Nanjing	44 (16.4)	35 (17.5)	
	MTHFR C677T		
CC	64 (23.9)	50 (25.0)	0.880
CT	139 (51.9)	99 (49.5)	
TT	65 (24.3)	51 (25.5)	
	MTHFR A1298C		
AA	190 (70.9)	136 (68.0)	0.260
AC	72 (26.9)	54 (27.0)	
CC	6 (2.2)	10 (5.0)	
	MTR A2756G		
AA	222 (82.8)	160 (80.0)	0.367
AG	45 (16.8)	37 (18.5)	
GG	1 (0.4)	3 (1.5)	
	MTRR A66G		
AA	77 (28.7)	76 (38.0)	0.089
AG	146 (54.5)	91 (45.5)	
GG	45 (16.8)	33 (16.5)	

3.2. Associations between Genotypes and Folate Level

The associations between the genotypes and the serum folate level are shown in Table 3. The patients with the *MTHFR* 677TT genotype had a lower serum folate level than the 677CC carriers (adjusted β (SE): −0.27 (0.01), $p < 0.001$). However, there was no significant difference in folate levels between the 677CT and 677CC genotypes. When the 677CC and 677CT genotypes were grouped together, we found that the 677TT carriers had a lower serum folate level than the 677CC + CT carriers (adjusted β (SE): −0.19 (0.02), $p < 0.001$). However, the patients with the *MTHFR* 1298AC + CC genotypes had a higher serum folate level than those with the wild-type genotype (adjusted β (SE): 0.10 (0.02), $p = 0.025$). Furthermore, the patients with *MTR* 2756AG + GG had a lower serum folate level

than the 66AA carriers (adjusted β (SE): −0.12 (0.02), $p = 0.005$). There was no significant correlation between the *MTRR* A66G polymorphism and serum folate.

Table 3. Associations of gene polymorphisms with serum folate level.

Genotype	Folate [1]	log(Folate) [1]	Crude		Adjusted [2]	
			β (SE)	*p*	β (SE)	*p*
MTHFR C677T						
CC ($n = 114$)	14.97 ± 6.23	1.14 ± 0.16	Reference		Reference	
CT ($n = 238$)	14.00 ± 6.16	1.11 ± 0.17	−0.09 (0.02)	0.098	−0.07 (0.02)	0.186
TT ($n = 116$)	11.85 ± 5.22	1.04 ± 0.16	−0.30 (0.01)	<0.001	−0.27 (0.01)	<0.001
CC + CT ($n = 352$)	14.32 ± 6.19	1.12 ± 0.17	Reference		Reference	
TT ($n = 116$)	11.85 ± 5.22	1.04 ± 0.16	−0.21 (0.02)	<0.001	−0.19 (0.02)	<0.001
MTHFR A1298C						
AA ($n = 326$)	13.35 ± 5.81	1.09 ± 0.16	Reference		Reference	
AC ($n = 126$)	14.62 ± 6.61	1.13 ± 0.17	0.10 (0.02)	0.039	0.10 (0.02)	0.026
CC ($n = 16$)	13.73 ± 6.15	1.10 ± 0.18	0.01 (0.02)	0.818	0.03 (0.02)	0.596
AA ($n = 326$)	13.35 ± 5.81	1.09 ± 0.16	Reference		Reference	
AC + CC ($n = 142$)	14.52 ± 6.54	1.13 ± 0.17	0.09 (0.02)	0.049	0.10 (0.02)	0.025
MTR A2756G						
AA ($n = 382$)	13.95 ± 6.26	1.11 ± 0.17	Reference		Reference	
AG + GG ($n = 86$)	12.61 ± 4.96	1.07 ± 0.17	−0.10 (0.02)	0.041	−0.12 (0.02)	0.006
MTRR A66G						
AA ($n = 153$)	13.57 ± 5.78	1.10 ± 0.17	Reference		Reference	
AG ($n = 237$)	13.83 ± 5.60	1.11 ± 0.16	0.04 (0.02)	0.480	0.02 (0.02)	0.731
GG ($n = 78$)	13.61 ± 7.76	1.09 ± 0.19	−0.04 (0.01)	0.600	−0.03 (0.01)	0.665
AA ($n = 153$)	13.57 ± 5.78	1.10 ± 0.17	Reference		Reference	
AG + GG ($n = 315$)	13.77 ± 6.19	1.10 ± 0.17	0.02 (0.02)	0.727	0.01 (0.02)	0.841

[1] Data are presented as the mean ± standard deviation (nmol/L). [2] Adjusted for sex, age, clinical center, height, and weight.

The effects of the four genotypes on folate deficiency are shown in Table 4. Using the *MTHFR* 677CC + CT genotypes as references, the odds ratio of the 677TT carriers was 2.34 (95% CI 1.47–3.71, $p < 0.001$). Additionally, the patients with the *MTR* 2756AG + GG genotypes had a higher risk of folate deficiency than the 2756AA carriers (OR = 1.80, 95% CI 1.06–3.05, $p = 0.030$). However, the patients with *MTHFR* 1298AC + CC showed a lower risk of folate deficiency, although this finding did not reach significance. Additionally, we found no significant effect of the *MTRR* A66G polymorphism on folate deficiency.

3.3. Gene-Gene Interactions on Folate Deficiency

We next examined the joint effects of these four gene polymorphisms on deficiency (Table 5). None of the patients had the 677TT/1298AC + CC genotypes, consistent with some previous reports [21,22]. Haplotypes of these two mutations have been suggested to be in complete linkage disequilibrium (p-value < 0.0001) [26]. Compared with the 677CC + CT/1298AC + CC carriers, the patients with the 677TT/1298AA genotypes had higher odds of folate deficiency (OR = 2.45, 95% CI 1.41–4.28, $p = 0.002$). Furthermore, the patients with 677TT/2756AG + GG had a four-fold increased risk of folate deficiency compared with those carrying 677CC + CT/2756AA (OR = 4.13, 95% CI 1.68–10.13, $p = 0.002$), and the 677TT/66AG + GG carriers had higher odds of folate deficiency than the 677CC + CT/66AA carriers (OR = 2.50, 95% CI 1.33–4.67, $p = 0.004$). Additionally, the 1298AA/2756AG + GG and 2756AG + GG/66AG + GG carriers both had higher risks of folate deficiency compared with the reference group. We further performed the trend test to verify these findings and, except for the *MTHFR* A1298C/*MTRR* A66G combination, the other genotype combinations dramatically increased the folate deficiency risk.

Table 4. Effects of gene polymorphisms on folate deficiency.

Genotype	Low folate [1]	High folate [1]	OR (95%Cl)	p [2]
		MTHFR C677T		
CC	26 (19.8)	88 (26.1)	Reference	
CT	57 (43.5)	181 (53.7)	1.01 (0.58–1.74)	0.980
TT	48 (36.6)	68 (20.2)	2.35 (1.29–4.26)	0.005
CC + CT	83 (63.4)	269 (79.8)	Reference	
TT	48 (36.6)	68 (20.2)	2.34 (1.47–3.71)	<0.001
		MTHFR A1298C		
AA	98 (74.8)	228 (67.7)	Reference	
AC	27 (20.6)	99 (29.4)	0.61 (0.37–1.01)	0.055
CC	6 (4.6)	10 (3.0)	1.18 (0.40–3.48)	0.770
AA	98 (74.8)	228 (67.7)	Reference	
AC + CC	33 (25.2)	109 (32.3)	0.67 (0.42–1.07)	0.095
		MTR A2756G		
AA	100 (76.3)	282 (83.7)	Reference	
AG + GG	31 (23.7)	55 (16.3)	1.80 (1.06–3.05)	0.030
		MTRR A66G		
AA	41 (31.3)	112 (33.2)	Reference	
AG	60 (45.8)	177 (52.5)	0.99 (0.61–1.60)	0.966
GG	30 (22.9)	48 (14.2)	1.65 (0.90–3.01)	0.105
AA	41 (31.3)	112 (33.2)	Reference	
AG + GG	90 (68.7)	225 (66.8)	1.14 (0.72–1.79)	0.579

[1] Data are presented as the number of cases (percentage). Low folate: serum folate level < 10 nmol/L; high folate: serum folate level $\geq$ 10 nmol/L. [2] Adjusted for sex, age, clinical center, height, and weight.

We used the GMDR model to explore the effects of high-order gene-gene interactions on folate deficiency (Table 6). The model with *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G had the highest training balance accuracy (0.6357), a relatively high testing balance accuracy (0.5717), the maximum cross-validation consistency of 10/10, and a significant p value (p = 0.0107). Thus, this model is probably the best model for interpreting the effects of high-order gene-gene interactions on folate deficiency.

3.4. Cumulative Effects of Risk Genotypes on Folate Deficiency

We defined the risk genotypes as 677TT, 1298AA, 2756AG + GG, and 66AG + GG. The cumulative effects of these four polymorphisms on folate deficiency are shown in Table 7. Due to the numbers of patients with all these four risk genotypes being relatively low, we combined the patients carrying three or four risk genotypes for evaluation. Compared with the null risk genotype carriers, the patients carrying three risk genotypes had a higher folate deficiency risk, with an odds ratio of 2.53 (95% CI 1.10–5.85; p = 0.029). In addition, the patients with four risk genotypes had a nearly four-fold increased risk of folate deficiency (OR = 3.77, 95% CI 1.07–13.27; p = 0.039). When we combined the patients with three or four risk genotypes, the increase in the folate deficiency risk remained significant (OR = 2.68, 95% CI 1.18–6.09; p = 0.019). Furthermore, the trend test showed a dramatic increase in folate deficiency with an increase in the number of risk genotypes (p_{trend} = 0.001). Plausibly, these results further support the conclusion drawn from the GMDR results that potential interactions among these gene polymorphisms may affect the incidence of folate deficiency.

Table 5. Effects of gene-gene interactions on folate deficiency.

Genotype 1	Genotype 2	N_L/N_H [1]	OR (95%Cl)	p [2]	Trend test	p [2]
MTHFR C677T	*MTHFR* A1298C					
CC + CT	AC + CC	33/109	Reference			
CC + CT	AA	50/160	1.09 (0.65–1.82)	0.757	0.16 (0.02)	<0.001
TT	AC + CC	0/0	-	-		
TT	AA	48/68	2.45 (1.41–4.28)	0.002		
MTHFR C677T	*MTR* A2756G					
CC + CT	AA	65/225	Reference			
CC + CT	AG + GG	18/44	1.69 (0.88–3.23)	0.116	0.19 (0.02)	<0.001
TT	AA	35/57	2.22 (1.32–3.74)	0.003		
TT	AG + GG	13/11	4.13 (1.68–10.13)	0.002		
MTHFR C677T	*MTRR* A66G					
CC + CT	AA	29/91	Reference			
CC + CT	AG + GG	54/178	1.02 (0.60–1.73)	0.954	0.15 (0.02)	0.001
TT	AA	12/21	2.03 (0.84–4.87)	0.114		
TT	AG + GG	36/47	2.50 (1.33–4.67)	0.004		
MTHFR A1298C	*MTR* A2756G					
AC + CC	AA	26/92	Reference			
AC + CC	AG + GG	7/17	2.07 (0.72–5.92)	0.177	0.11 (0.02)	0.019
AA	AA	74/190	1.50 (0.89–2.54)	0.131		
AA	AG + GG	24/38	2.54 (1.26–5.15)	0.010		
MTHFR A1298C	*MTRR* A66G					
AC + CC	AA	13/38	Reference			
AC + CC	AG + GG	20/71	0.87 (0.38–1.99)	0.743	0.08 (0.02)	0.088
AA	AA	28/74	1.18 (0.54–2.60)	0.680		
AA	AG + GG	70/154	1.46 (0.72–2.98)	0.297		
MTR A2756G	*MTRR* A66G					
AA	AA	30/93	Reference			
AA	AG + GG	70/189	1.16 (0.69–1.94)	0.570	0.10 (0.02)	0.032
AG + GG	AA	11/19	1.73 (0.70–4.29)	0.240		
AG + GG	AG + GG	20/36	2.09 (1.01–4.29)	0.046		

[1] N_L: number of low folate patients (<10 nmol/L); N_H: number of high folate patients (≥10 nmol/L); [2] Adjusted for sex, age, clinical center, height, and weight.

Table 6. GMDR models of effects of high-order interactions on folate deficiency.

Models [1]	Training balance accuracy	Testing balance accuracy	Sign test (p value)	Cross-validation consistency
C677T	0.5842	0.5825	8 (0.0547)	10/10
C677T, A2756G	0.6047	0.5946	10 (0.0010)	10/10
C677T, A2756G, A66G	0.6236	0.5631	10 (0.0010)	6/10
C677T, A2756G, A1298C, A66G	0.6357	0.5717	9 (0.0107)	10/10

[1] All models adjusted for sex, age, clinical center, height and weight.

Table 7. Cumulative effects of risk genotypes [1] on folate deficiency.

Number of risk genotypes	Low folate [2]	High folate [2]	OR (95%CI)	p [3]	p_{trend} [3]
0	10 (7.6)	33 (9.8)	Reference		
1	32 (24.4)	106 (31.5)	1.01 (0.44–2.32)	0.989	
2	40 (30.5)	132 (39.2)	1.17 (0.52–2.63)	0.710	0.001
3	41 (31.3)	58 (17.2)	2.53 (1.10–5.85)	0.029	
4	8 (6.1)	8 (2.4)	3.77 (1.07–13.27)	0.039	
≥3	49 (37.4)	66 (19.6)	2.68 (1.18–6.09)	0.019	

[1] Risk genotypes were defined as *MTHFR* 677TT, *MTHFR* 1298AA, *MTR* 2756AG + GG, and *MTRR* 66AG + GG. [2] Data are presented as the number of cases (percentage). Low folate: serum folate level <10 nmol/L; high folate: serum folate level ≥10 nmol/L. [3] Adjusted for sex, age, clinical center, height, and weight.

4. Discussion

In the present study, the *MTHFR* C677T and *MTR* A2756G polymorphisms each independently reduced the serum folate level and increased the folate deficiency risk. In addition, compared with the wild-type of *MTHFR* A1298C genotype, patients carried the mutant C allele showed higher folate level. We defined the risk genotypes as *MTHFR* 677TT, *MTHFR* 1298AA, *MTR* 2756AG + GG, and *MTRR* 66AG + GG. Assessment of gene-gene interactions using the ULR and GMDR models revealed significant effects of interactions of these four risk genotypes on folate deficiency.

Folate is a crucial factor in cell division and cell maintenance and also plays an important role in regulating epigenetic gene expression [12]. A recent study has shown that three forms of folate supplementation (natural folate-rich foods, folic acid and 5-MTHF) have similar effects on lowering plasma homocysteine [8]. Pravenec *et al.* have indicated that a reduction in the folate level aggravates evidence of oxidative tissue damage and insulin resistance, and elevates blood pressure in spontaneously hypertensive rats [27]. We found that a low folate level resulted in a significant increase in the plasma homocysteine concentration ($p < 0.001$, data not show) and this negative correlation has been widely confirmed [10,23,25,28]. Furthermore, several studies showed that folate supplementation reduced the plasma homocysteine level, urinary 8-iso-prostaglandin $F_{2\alpha}$ and 11-dehydro-thromboxane B_2 excretion, and increased serum folate level especially in hyperhomocysteinemic carriers [29,30]. These results showed the beneficial of folate supplementation on oxidative stress and platelet activation. In addition, a prospective study has shown that folate deficiency is independently predictive of a 53% increased risk of coronary heart disease mortality in older adults [31]. In an Indian cohort, the *MTHFR* 677T allele and folate deficiency independently increased neonatal hyperbilirubinemia risk by approximately four-fold and three-fold, respectively [32]. These findings indicate that folate deficiency may independently increase the risk of CVDs.

According to the threshold of 10 nmol/L, approximately 28% (131) of the Chinese hypertension patients in our study were folate deficient. Toprak *et al.* reported only 201 (1.1%) subjects with a folate level of $\leq$5 nmol/L and 640 (4.7%) with a level of $\leq$6.8 nmol/L out of a total of 17,713 participants [33], suggesting that use of a higher folate cutoff value may improve sensitivity for detecting a deficiency. The relationship between folate and homocysteine may differ depending on whether the folate level is low or high. Selhub *et al.* have reported that the serum homocysteine concentration increases as the serum folate concentration decreases; however, at a high folate level, this correlation may disappear [25]. In addition, the two-phase regression model suggests that the threshold folate level is approximately 10 nmol/L, which is the level at which the homocysteine concentration approaches flat [25]. Therefore, in this study, we defined folate deficiency as a serum folate level of <10 nmol/L.

We observed that the patients with *MTHFR* 677TT had a lower serum folate level ($p < 0.001$, Table 3) and a higher odds of folate deficiency ($p < 0.001$, Table 4) than the 677CC + CT carriers. This negative correlation between *MTHFR* C677T polymorphisms and the folate level has been widely reported [7,10,11,19,34]. A previous study has reported that *MTHFR* 677TT may cause an approximately 70% decrease and 677CT results in a 35% decrease in the mean MTHFR activity [35]. The *MTHFR* C677T mutation is located in the catalytic domain of the enzyme [35,36] and causes an alanine to valine substitution at position 222, resulting in a thermolabile enzyme [22,35]. The homozygous *MTHFR* C677T mutation decreases enzymatic activity and causes a lower rate of reduction of 5, 10-methylene-THF to 5-methyl-THF, resulting in increased availability of 5, 10-methylene-THF for oxidation to the formylated folate forms and accumulation in red blood cells (RBCs) [37]. However, no formylated folates have been found in RBCs of 677CC genotype carriers. Furthermore, because mature RBCs have almost no ability to transport folate, accumulation of formylated-THF RBCs may lead to lower proportions of 5, 10-methylene-THF and 5-methyl-THF in the serum, which could explain the decreased serum folate level observed in individuals with the 677TT genotype. However, Waskiewicz *et al.* failed to find this association in either adult Polish men or women [38]. Moreover, a low folate level and *MTHFR* 677TT may have a synergistic effect on elevating the plasma homocysteine concentration [10,11]. *MTHFR* A1298C is located in the regulatory domain named NADPH and

S-adenosylmethionine binding site [36]. The A1298C mutation does not result in synthesis of a thermolabile protein [22]. Thus, this mutation may not have a significant effect on the serum folate level (AA *vs.* AC + CC, $p = 0.684$) [7]. Further, Yakub *et al.* have not found an effect of *MTHFR* A1298C on the serum folate level [10]. Our results showed that heterozygous and homozygous A1298C mutations resulted in a higher folate level compared with that resulting from the wild-type genotype (adjusted $p = 0.025$, Table 3). Furthermore, *MTHFR* 1298AC + CC may be a protective factor that reduces the risk of folate deficiency, although this finding was not significant (Table 4). Similar to our results, Biselli *et al.* have found that the mean plasma folate concentration is significantly higher in carriers of the altered allele (1298AC and 1298CC) compared with carriers of 1298AA in coronary artery disease patients [18]. One possible reason for this finding is that S-adenosylmethionine is an allosteric inhibitor of MTHFR [36]. Structural prediction revealed that the S-adenosylmethionine binding site in the mutated structure is distorted compared with that in the normal structure [36] and therefore, it may reduce the inhibitory effect of the enzyme. However, in contrast with the results of our study, subjects carrying the 1298CC genotype have been demonstrated to have a lower serum folate level compared with the levels in 1298AC and 1298CC carriers in a study of male clear cell renal cell carcinoma patients and control individuals [19]. Therefore, the potential impact of *MTHFR* A1298C on the serum folate level needs more investigation.

The physiologically active coenzyme form of folate is tetrahydrofolate (THF). *In vivo*, THF is converted into 5, 10-methylene-THF with the aid of vitamin B_6 and then into 5-methyl-THF. The conversion of 5, 10-methylene-THF into 5-methyl-THF is physiologically irreversible [25]. Therefore, reduced MTR activity may decrease the rate of conversion of 5-methyl-THF to THF, then results in physiological folate deficiency. *MTR* A2756G mutation located at position 919 of the protein results in substitution of glycine for aspartic acid [39]. It is located in a domain of the protein that interacts with S-adenosylmethionine and auxiliary proteins that are required for the reductive methylation and reactivation of the vitamin B_{12} cofactor, which can be inactivated by oxidation during catalysis [17,40]. Therefore, this mutation might impair the binding of SAM and/or auxiliary proteins [40] and reduce catalytic efficiency. In this study, we found that the *MTR* 2756AG + GG genotypes resulted in not only a decreased serum folate level ($p = 0.006$, Table 3) but also an increased folate deficiency risk compared with the wild-type genotype ($p = 0.030$, Table 4). In contrast, studies of the Brazilian [7], Pakistani [10], and Jamaican [20] populations have failed to demonstrate a correlation between the *MTR* A2756G polymorphism and folate level. However, serum folate and the *MTHFR* 677CC+CT and *MTR* 2756AA genotypes have been shown to have significant interactions with total homocysteine in pregnant women [7]. Furthermore, in individuals with low intake of folate, vitamin B_6, and vitamin B_{12}, the *MTHFR* 677T and *MTR* 2756G alleles have been shown to result in a high risk of breast cancer [41].

A common *MTRR* polymorphism is the substitution of A for G at nucleotide 66, which results in the substitution of isoleucine by methionine. This mutation is located in the putative flavin mononucleotide-binding domain of the MTRR enzyme, which interacts with MTR [17] and, thus, disrupts the binding of MTRR to the MTR-cobalamin-complex, decreasing the rate of homocysteine remethylation [39]. Our results showed that *MTRR* 66AG + GG may not affect the serum folate level or the incidence of folate deficiency (Tables 3 and 4). Similarly, Feix *et al.* have reported that the *MTRR* A66G polymorphism has no effects on the total homocysteine, folate or vitamin B_{12} concentrations [42]. Although the *MTRR* A66G polymorphism has no significant influence on the serum folate level, the combination of *MTHFR* C677T and *MTRR* A66G have significant interactive effects on the total homocysteine and serum folate concentrations [7].

Based on these findings, we hypothesized that multiple genetic defects will aggravate folate deficiency. Several studies showed the combination effect of *MTHFR* C677T and A1298C decreased serum folate level [7,19,22]. MTHFR is crucial for maintaining an adequate methionine pool and for ensuring that the homocysteine concentration does not reach a toxic level [43]. The homozygous *MTHFR* C677T mutation causes a decrease in the conversion rate of 5, 10-methylene-THF to 5-methyl-THF resulting in a reduction in the 5-methyl-THF level [35]. Subsequently, the supply

of methyl groups is diminished, affecting the synthesis of methionine from homocysteine. *MTR* 2756G allele affects the binding of accessory proteins involved in cofactor reduction [44], potentially resulting in reductions in the synthesis rates of 5-methyl-THF to THF and homocysteine to methionine. A low THF level also affects folate circulation. Furthermore, MTRR restores the activity of MTR [17]. Thus, a mutation in MTRR may result in a decreased recovery rate of the oxidant cobalamin, indirectly affecting the serum folate level. As such, the deleterious effects of homocysteine metabolism gene polymorphisms on serum folate level are biologically plausible. In Brazilian children, the folate level has been shown to be significantly decreased in subjects with the 677CC/1298AA/66AA genotypes compared with those carrying 677CC/1298AA/66AG ($p = 0.03$), 677CC/1298AC/66AG ($p = 0.003$) and 677CT/1298AA/66AG ($p = 0.02$) [21]. In our study, the assessment of high-order gene-gene interactions with our GMDR model revealed that all four gene polymorphisms interactively influenced the prevalence of folate deficiency (Table 6). The results from the evaluation of cumulative effects verified these results (Table 7).

To the best of our knowledge, we are the first to demonstrate that the *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G gene polymorphisms have significant interactive effects on the risk of folate deficiency in Chinese hypertensive patients. A limitation of our study is that the sample size was relatively small. Therefore, we believe that future studies with large samples could be performed to validate our results in a more expansive population.

5. Conclusions

Folate deficiency is a risk factor for cardiovascular disease that is modified by several gene polymorphisms. This study showed that *MTHFR* 677TT, *MTHFR* 1298AA, and *MTR* 2756AG + GG are independently correlated with a high risk of folate deficiency. Furthermore, we have demonstrated that not only pairwise gene-gene interactions but also higher-order interactions of these gene polymorphisms (*MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G, and *MTRR* A66G) more strongly influence the incidence of folate deficiency. We suggest that individuals who carry those risk genotypes (especially for multiple risk genotypes carriers) should be monitored for their folate circulating levels, and have folate supplementation in case of deficiency. Whether this intervention may translate into a meaningful reduction of cardiovascular events will be hopefully unraveled in the next years.

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Quercetin Impacts Expression of Metabolism- and Obesity-Associated Genes in SGBS Adipocytes

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Abstract: Obesity is characterized by the rapid expansion of visceral adipose tissue, resulting in a hypoxic environment in adipose tissue which leads to a profound change of gene expression in adipocytes. As a consequence, there is a dysregulation of metabolism and adipokine secretion in adipose tissue leading to the development of systemic inflammation and finally resulting in the onset of metabolic diseases. The flavonoid quercetin as well as other secondary plant metabolites also referred to as phytochemicals have anti-oxidant, anti-inflammatory, and anti-diabetic effects known to be protective in view of obesity-related-diseases. Nevertheless, its underlying molecular mechanism is still obscure and thus the focus of this study was to explore the influence of quercetin on human SGBS (Simpson Golabi Behmel Syndrome) adipocytes' gene expression. We revealed for the first time that quercetin significantly changed expression of adipokine (Angptl4, adipsin, irisin and PAI-1) and glycolysis-involved (ENO2, PFKP and PFKFB4) genes, and that this effect not only antagonized but in part even overcompensated the effect mediated by hypoxia in adipocytes. Thus, these results are explained by the recently proposed hypothesis that the protective effect of quercetin is not solely due to its free radical-scavenging activity but also to a direct effect on mitochondrial processes, and they demonstrate that quercetin might have the potential to counteract the development of obesity-associated complications.

Keywords: quercetin; phytochemicals; enolase 2; ENO2; angiopoietin-like 4; ANGPTL4; plasminogen activator inhibitor-1; PAI-1; SERPINE1; phosopho-fructokinase; PFKP; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; PFKFB4; complement factor D; adipsin; CFD; fibronectin type III domain-containing 5; irisin; FNDC5; interleukin-1β; IL1B

1. Introduction

Obesity confers a high risk of developing numerous metabolic and cardiovascular complications. In the context of extensively growing or prevailing adipose tissue, biochemical and cellular changes take place in adipocytes, in the presence of reduced oxygen supply [1]. Hypoxia is a major starting point of the inflammatory process in adipose tissue and modulates adipocyte metabolism [2–5]. Insufficient oxygen supply is sensed via the mitochondrial electron transfer chain and thus reactive oxygen species (ROS) production is extremely elevated leading to stabilization of hypoxia inducible transcription factors [6], thereby taking over the key role in the activation of signaling pathways

that are relevant for further metabolic adaptation and adipokine secretion in adipocytes resulting in a dysfunction of adipose tissue [7]. There is evidence that this dysregulation of metabolism in adipose tissue under hypoxia promotes insulin resistance and dyslipidemia and consequently initiates the development of diabetes and cardiovascular disease [2,8].

For that reason, there is growing interest worldwide in plant compounds with respect to their potential to combat obesity and subsequent diseases. Among the more than 4000 flavonoids [9] quercetin is the most common one. Found in fruits, vegetables, wine, tea, and nuts, it represents a central part of our diet [10]. It is regarded as the most effective scavenger of ROS [11] as well. Molecular effects of this phytochemical are poorly understood, although the mitochondrial membrane seems to play a major role herein [10]. The present work was aimed at elucidating the effect of quercetin on the expression of adipokine, glycolytic, and inflammatory genes in hypoxic human Simpson Golabi Behmel Syndrome (SGBS) adipocytes.

2. Materials and Methods

2.1. Cell Culture and Reagents

Human SGBS preadipocytes were kindly provided by Dr. M. Wabitsch [12] and have been cultivated as described previously [12]. Briefly, cells were maintained in 15 mL DMEM/Ham's F12 (1:1) medium (Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS; Invitrogen), 100 U/mL penicillin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), 33 µM biotin, and 17 µM pantothenate. To differentiate SGBS cells into adipocytes, near confluent cells were washed three times with phosphate buffered saline (PBS) and cultured in FCS-free differentiation medium: DMEM/Ham's F12 (1:1) medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 33 µM biotin, 17 µM pantothenate, 10 µg/mL human transferrin, 10 nM insulin, 100 nM hydrocortisone, 0.2 nM triiodothyronine, 25 nM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine (IBMX), and 2 µM rosiglitazone. After four days, this medium was replaced by differentiation medium excluding dexamethasone, IBMX, and rosiglitazone, which was changed every three to four days. At day 12 after induction of differentiation, 25 µM quercetin (Q4951-10G, LOT#SLBD8415V, Sigma-Aldrich, Steinheim, Germany), dissolved in 23 µL DMSO, was added to cell cultures (15 mL), and 23 µL DMSO without quercetin was added to control-cultures (15 mL), resulting in a 0.15% (*v*/*v*) DMSO concentration in all cell cultures. All cell cultures were cultivated for another 48 h and then exposed to hypoxia. To create a hypoxic environment (1% O_2), cells were placed in a MIC-101 modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA), flushed with a mixture of 1% O_2, 5% CO_2, and 94% N2, sealed, and incubated at 37 °C. Adipocytes were cultured in hypoxic environment for 16 h, whereas control groups were cultured under normoxic conditions (21% O_2). In total we had four different treatment groups, and each of these approaches consisted of four independent experiments. Reagents were obtained from Sigma-Aldrich unless specified otherwise.

2.2. Cell Lysis

Total RNA was prepared from cell samples using the RNeasy Lipid Tissue kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), including the optional DNase step. RNA quantity and purity was determined on NanoDropTM 2000 (Thermo Scientific, Waltham, MA, USA).

2.3. Quantitative PCR

RNA was reverse transcribed using the SuperScript III First-Strand Synthesis Kit (Invitrogen) and quantitative PCR (qPCR), was performed using the PowerSYBR® Green PCR Master Mix (Thermo Scientific) on LightCycler® 480 System (Roche Diagnostics, Rotkreuz, Switzerland). The primers were synthesized by Microsynth (Balgach, Switzerland) (sequences are disclosed in the supplementary section). A melting curve profile was processed after each run to confirm specific transcripts.

All reactions were performed in triplicates and the samples were normalized to the endogenous reference TATA-binding protein (TBP) values.

2.4. Data Analysis

Results were calculated as cycle threshold values relative to controls according to the ΔΔCt method and expressed as fold change (FC, $2^{-\Delta\Delta Ct}$). Standard deviation of FC (SD) has been calculated according to range for target relative to calibrator resulting from incorporating the standard deviation s of the ΔΔCt values into the fold-difference calculation: $2^{-\Delta\Delta Ct}$ with ΔΔCt + s, and ΔΔCt − s. Statistical data analysis was performed using IBM SPSS (version 22, Armonk, NY, USA). Normal distribution of data was confirmed by the Shapiro-Wilks test. To test for significant differences, we used one-way ANOVA to see if there were between-group differences. As there were significant differences in all cases, we proceeded to *post-hoc* testing by multiple comparison Bonferroni testing. *p*-values smaller than 0.05 were considered significant.

3. Results

In the present study we focused on the expression of genes which are known or suspected to be impacted by a hypoxic environment in adipose tissue. In addition, genes were selected based on (i) their role in glucose metabolism and inflammation; (ii) their function as adipokines; and (iii) their role in obesity-associated diseases such as diabetes. These genes were enolase 2 (ENO2), angiopoietin-like 4 (ANGPTL4), plasminogen activator inhibitor-1 (PAI-1, also known as SERPINE1), platelet-type 6-phosophofructokinase (PFKP), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4), complement factor D (CFD, also known as adipsin), fibronectin type III domain-containing 5 (FNDC5, which encodes the precursor of irisin), and interleukin-1β (IL1B). Expression of the respective genes was assessed in differentiated, mature SGBS adipocytes, which were cultivated at 37 °C under normoxia (N) or hypoxia (H) in media supplemented with (Q) or without 25 μM quercetin (C) for 16 h. Results of gene expression analysis for the four treatment groups (CN, QN, CH, and QH) are summarized in Figure 1 and the supplementary section.

For each gene, mRNA levels were assessed to investigate the effect of quercetin under normoxic cultivation (QN) compared to normoxic cultivation without quercetin (CN), the effect of hypoxic cultivation without quercetin (CH) compared to normoxic cultivation without quercetin (CN), the effect of both quercetin supplementation and hypoxic cultivation (QH) compared to normoxic cultivation without quercetin (CN), and the effect of quercetin under hypoxic cultivation (QH) compared to hypoxic cultivation without quercetin (CH, see supplementary section).

Cultivation of the SGBS adipocytes under hypoxia significantly increased expression of ENO2, PFKP, and PFKFB4 compared to cultivation under normoxia. No significant effect of hypoxia was seen for gene expression of FNDC5/irisin, of adipokines PAI-1 and CFD/adipsin, nor of IL-1β (see supplementary data), though expression of ANGPTL4 was just failing significance ($p = 0.057$). In contrast, after the supplementation of normoxic cultivated culture samples with 25 μM quercetin, we observed a significant decrease in the expression of ANGPTL4, CFD/adipsin, PAI-1, and PFKP, compared to normoxic cultivation without quercetin. In samples cultivated under hypoxia and supplemented with quercetin, compared to samples cultivated under hypoxia but without quercetin, we observed a significant decrease in ANGPTL4, CFD/adipsin, PAI-1, and PFKP, and additionally in PFKFB4 and ENO2. The strongest impact of quercetin supplementation, both under normoxic as well as hypoxic conditions, was observed for PFKP, as indicated by a 6.5 fold decrease of gene expression under normoxia (FC = 0.155; $p = 6.9 \times 10^{-6}$) and a 9.2 fold decrease under hypoxia (FC = 0.109, $p = 2.6 \times 10^{-6}$). When comparing gene expression of quercetin-treated and hypoxic cultivated samples to samples cultivated under normoxia and without quercetin, we still found a significant inhibition of ANGPTL4, CFD/adipsin, PAI-1, and PFKP, whereas FNDC5/irisin, ENO2, and PFKFB4 gene expression was significantly raised instead.

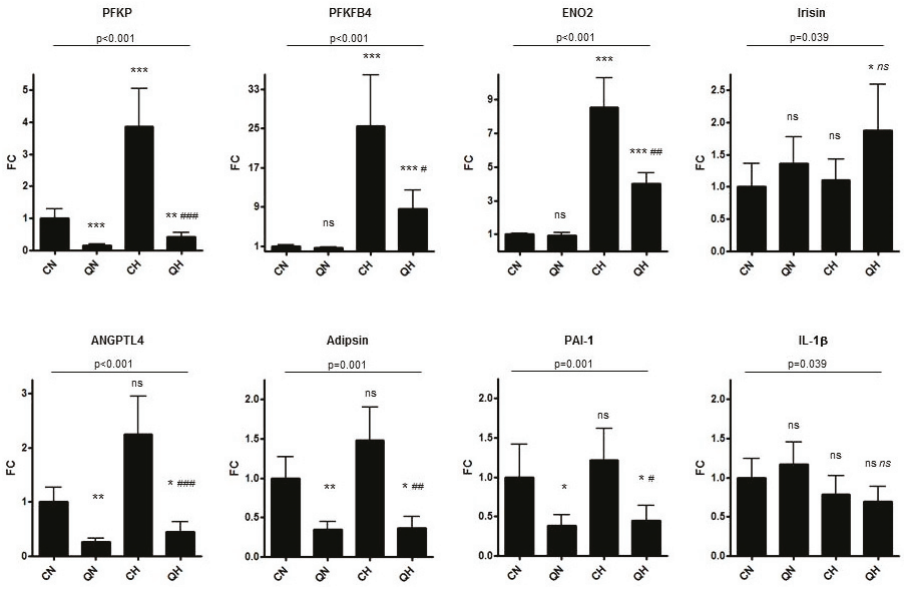

Figure 1. Impact of quercetin on gene transcription of normoxic and hypoxic adipocytes. Levels of mRNA were assessed in SGBS adipocytes cultivated in the presence (**Q**) or absence of quercetin (**C**) under normoxic (**N**) or hypoxic (1% O_2) conditions (**H**). Transcriptional alterations are expressed as fold change (**FC**) with standard deviation ($2^{-\Delta\Delta ct}$ with $\Delta\Delta ct + s$ and $\Delta\Delta ct - s$, where s is the standard deviation of the $\Delta\Delta ct$ value) relative to cultivation without quercetin in a normoxic atmosphere (CN). All data represent the mean of four independent experiments, each consisting of triplicates. TATA binding protein (TBP) has been used as a reference gene. According to one-way ANOVA, there were significant differences for all gene expression sets with respect to the four treatment groups (*p*-values are indicated). For analyzing differences between two treatment groups post hoc analysis according to Bonferroni was used. A *p*-value < 0.05, is marked as *, a *p*-value < 0.01 as **, and a *p*-value < 0.001 as *** for the comparison of QN, CH, or QH to CN. For the comparison of QH to CH a *p*-value < 0.05 is marked as #, a *p*-value < 0.01 as $^{\#\#}$, and a *p*-value < 0.001 as $^{\#\#\#}$.

4. Discussion

This work examined the regulatory impact of quercetin on the gene expression of human SGBS adipocytes. We demonstrated that quercetin is able to significantly decrease gene expression of adipokines ANGPTL4, adipsin, and PAI-1 as well as of glycolysis-associated enzymes ENO2, PFKP, and PFKFB4. Each of these is assumed to be involved in the development of obesity-associated complications.

The most striking effect was observed on the platelet-type 6-phosphofructokinase gene PFKP. It is involved in glycolysis, catalyzing fructose 6-phosphate to fructose1,6-bisphophate conversion. Elevated PFKP expression is known to be associated with increased body mass index (BMI) and obesity [13,14]. PFKP enzyme activity is inhibited by ATP, citrate, fatty acids [15], and by new synthetic molecules presently undergoing clinical trials [16,17]. We could clearly demonstrate that its expression is upregulated by hypoxia, which is, most likely, due to a direct binding of HIF-1α [3] and downregulated by quercetin, whereby the latter effect was predominant when both factors were applied in parallel.

The enolase 2 gene ENO2 is directly involved in glycolysis, catalyzing the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate. Similar to PFKP, ENO2 gene expression was significantly decreased by quercetin treatment under hypoxic conditions, but in contrast to PFKP, the attenuation by quercetin could not outperform the hypoxia-effect. The same applies to the expression of

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase gene PFKFB4. It regulates the steady-state concentration of 2,6-bisphosphate, an allosteric activator of phosphofructokinase. Like PFKP and ENO2, it is activated by hypoxia as well [3,18–20]. In metabolic screens PFKFB4 has been proposed as a new potential target in cancer therapy as its silencing increased the ROS level and inhibited survival of cancer cells but not epithelial cells. Thus PFKFB4 seems to be essential to keep balance between glycolytic activity and antioxidant production at least in cancer cells [21]. Whether hypoxia-mediated upregulation of PFKFB4 might prevent ROS overproduction in adipocytes is unknown, but appears contradictory.

The fasting induced adipose factor ANGPTL4 is predominantly produced in adipose tissue. It is a target of peroxisome proliferator-activated receptor (PPAR) γ [22] and recently it has been demonstrated to be inhibited by AMP-activated kinase (AMPK) activation [23,24]. It is an important player in energy metabolism and insulin sensitivity and its overexpression elevates triglycerides and total cholesterol and impacts the activity of mitochondrial respiratory chain complexes [25]. Although its role in the context of metabolic diseases is still elusive, ANGPTL4 plasma levels have been reported to be significantly higher in patients with metabolic syndrome and were predictive for future cardiovascular events [26]. In the present study, our data demonstrated a trend for hypoxia-mediated upregulation. Quercetin treatment, in contrast, led to a significant decrease of ANGPTL4 expression and that inhibiting effect was not abolished even in the presence of hypoxia.

Similarly, PAI-1 is also a target of PPARγ [27,28]. Quercetin has previously been described to activate AMPK and to decrease PPARγ expression [29]. AMPK activation is known to result in PPARγ inhibition [30–32]. As quercetin also exhibits the ability to decrease ATP production, causing an increase in AMP and the activation of the AMPK signaling pathway [33], a common regulatory mechanism for ANGPTL4 and PAI-1 appears evident. It is presently known that circulating PAI-1 levels are increased in the metabolic syndrome as well, and that they are strongly associated with visceral adiposity and may contribute to the inflammatory state in obesity [34]. Moreover, mice lacking PAI-1 have increased energy expenditure, improved glucose tolerance, enhanced insulin sensitivity, and are resistant to diet- or genetically induced obesity. Improvement of insulin sensitivity by weight loss or treatment with insulin sensitizers such as metformin or thiazoladinediones significantly reduces circulating PAI-1 levels [34]. Thus PAI-1 has been considered as a biomarker to predict obesity-associated diseases [35]. Apart from the role as a marker, the PAI-1-PPARγ interaction may also be a potential target for novel anti-obesity drugs. In that context, our findings, which indicate that there is a significant downregulation of PAI-1 expression upon quercetin treatment independent of normoxic or hypoxic cultivation, together with the previously reported PAI-1 downmodulation by resveratrol [36], may be helpful in elucidating the detailed mode of action of these phytochemicals for future clinical use.

Knowledge about the adipokine adipsin, which is encoded by the CFD gene, is very limited. It is the rate-limiting enzyme of the alternative complement pathway, working as serine protease [37], and it is mainly produced by adipocytes [38]. It is known that adipsin levels are associated with BMI, but the way its expression is regulated is presently unknown [39]. In the context of macular degeneration, a decrease of adipsin [40] and an inhibition of the systemic activation of the complement system [41] have both been observed in the plasma of affected patients upon treatment with the anti-oxidant lutein. Whether that anti-oxidant affects gene expression of CFD/adipsin in adipocytes is not known [39]. Here, we are able to describe for the first time a downmodulation of CFD/adipsin by the anti-oxidant quercetin in adipocytes. Thus these results are of broad clinical interest.

FNDC5, primarily identified as a myokine which is cleaved and secreted as irisin from muscle during exercise is known to induce metabolic benefits after exercise [42]. A recent study reported that white adipose tissue in humans and rats is able to express and secrete FNDC5/irisin as well [43]. In line with data from a human trial reporting no effect of hypoxia on irisin levels [44], we also did not observe a significant effect of hypoxic treatment on the expression of FNDC5/irisin in our study. Interestingly, we observed a significant increase of FNDC5/irisin expression by quercetin supplementation under hypoxia and there is, to our knowledge, no other study reporting the impact of phytochemicals on FNDC5/irisin in adipocytes. Of interest, FNDC5 expression is, in contrast to ANGPTL4 and PAI-1,

known to be elevated by AMPK activation [42]. In line with our data, a similar effect on FNDC5/irisin expression was seen in the hippocampus tissue of rats receiving quercetin leading to a protection against brain damage under hypoxia [45]. Thus these findings may together initiate future studies further elucidating the overall function of irisin in cell types other than myocytes.

We also observed that neither quercetin nor hypoxic cultivation did impact the expression of IL-1β. This is, in case of the latter, a bit surprising as hypoxia in adipose tissue is suggested to induce inflammation. However, similar results have been previously reported in human adipocytes [4,46]. Drawing conclusions from adipocytes about adipose tissue is problematic and does not take into account the complex *in vivo* situation with various cell-type interactions involved in triggering and regulating the inflammatory cascade. That issue has been reviewed in detail recently [47].

Quercetin has a radical scavenging capacity [48] and its pure stoichiometric consumption of free radicals by the molecule structure itself has the potential to antagonize hypoxia [49]. Hypoxia is sensed by mitochondria and leads to an increase in ROS generation [6]. ROS overproduction has previously been hypothesized to trigger a couple of independent pathways implicated in metabolism [50]. Such chronic oxidative stress plays a pivotal role in the pathogenesis of degenerative disorders [51]. On a molecular level, the rise of ROS levels leads to the stabilization of HIF-1 [6,52]. Hence the antioxidant capacity of quercetin might be responsible for a decreased expression of HIF-1-dependent genes like PFKFB4 [19], PFKP, and ENO2 [3]. This property of quercetin is in line with the reduced expression of PFKFB4 and ENO2 in our study and resembles the effect of a HIF-inhibitor [3]. However, it does not explain why gene expression in the case of PFKP or ANGPTL4 drops below the control level, outperforming by far the opposed effect of hypoxia. Likewise, the expression of CFD/adipsin and PAI-1 was significantly decreased as well, although hypoxia had only a slight and insignificant effect.

In order to tie all results together, we believe that quercetin action, which not only antagonizes but also outperforms the effect mediated by hypoxia, lies not only in its radical scavenging capacity, but is mainly based on intracellular mechanisms [33]. Quercetin is known to accumulate in mitochondria [53], it has previously been hypothesized by us [10] and has recently become more commonly accepted that quercetin influences the mitochondrial electron transfer chain [33,54]. In addition, this seems to be associated with quercetin's action on mitochondrial biogenesis and apoptosis but also on the mitochondrial permeability transition pore, the membrane potential, and finally ATP generation impacting the AMPK activity [29,45,55–63]. Of interest, Lago *et al.* have revealed complex I as a target of structural binding by quercetin competing with coenzyme Q [64]. Such a specific effect on the mitochondria may be associated with or causative for further downstream effects on different targets including AMPK activation. Thus the effects of quercetin as seen in our study may be related to its (i) radical scavenging activity which would appear to counteract hypoxia, but also to its (ii) direct binding of the mitochondrial electron transfer chain complexes, probably impacting mitochondrial function including energy homeostasis, which finally leads to the mentioned effects on AMPK-dependent targets.

Nevertheless, we have to mention that the detailed role of quercetin in hypoxic adipose tissue is still elusive and there are several mechanism including the inhibition of the proteasome [65], the modulation of the JNK and ERK pathway as well as AP-1 and NF-κB activation, and the ambiguous role of PPARγ [29,66] which need further investigation. Moreover, since the potential of human adipose tissue to differentiate is limited, SGBS adipocytes were used. They are a valuable, well established and relevant tool to study human adipocyte biology *in vitro* [12,67–73]. SGBS adipocytes are derived from the stromal cells fraction of subcutaneous adipose tissue of a patient suffering from Simpson-Golabi-Behmel syndrome, and feature a long lasting and high capacity for adipose differentiation and at the same time display a gene expression pattern similar to mature fat cells [12,74]. They have been used to explore the effect of hypoxia on adipose tissue [3,68,75–78] and a comprehensive gene expression and secretome profiling under hypoxic conditions has already been done [4,79]. However we cannot exclude that the underlying mutation in the SGBS cells coming

from the specimen of a diseased patient [67] may impact gene expression in a different way than it would be seen in adipocytes from a healthy subject. Therefore, additional studies using, for example, primary cells derived from lipoaspirates are needed to be performed to further elucidate the relation of whole adipose tissue hypoxia and the chronic inflammation observed in obesity.

Finally, the amount of quercetin used to treat SGBS adipocytes in the present study was 25 μM and that concentration was comparable to those from previous *in vitro* studies [10]. The estimated quercetin intake by Western diet ranges from 0 to 30 mg [80], whereas up to 2000 mg have been administered in clinical trials [10]. However, it has to be mentioned that quercetin bioavailability is low and varies widely between individuals due to endogenous and exogenous factors [81]. The utility of nanoparticles as delivery carriers for quercetin has been recently summarized by Nam *et al.* (2016) [82]. Such a nanoformulation demonstrates the ability to enhance solubility of quercetin in water, absorption into the body, circulation time, and target specificity. Thus these more stable and long-lived application forms may further release and potentiate quercetin's putative health benefits [82].

In conclusion, this study demonstrated that quercetin is able to antagonize and, in part, to overcome effects mediated by hypoxia. These results are in accordance with the hypothesis recently proposed by de Oliveira *et al.* suggesting that a direct free radical-scavenging activity of quercetin cannot be concluded as the major mechanism for the clinical effects of quercetin, and that there must be a direct effect in mitochondrial processes [33]. In addition, they further substantiate and elucidate quercetin's anti-diabetic effect and suggest that quercetin may play a protective role counteracting the development of obesity-associated associations.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/5/282/s1, Table S1: Oligonucleotide sequences. All sequences are given in 5′-3′ orientation, Table S2: Gene expression data according to qPCR analysis.

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Section 4: Functional Role of the Intestinal Micobiota in Metabolic Disorders

nutrients

MDPI

Review

Diet, Microbiota and Immune System in Type 1 Diabetes Development and Evolution

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Abstract: Type 1 diabetes (T1D) is the second most frequent autoimmune disease in childhood. The long-term micro- and macro-vascular complications of diabetes are associated with the leading causes of disability and even mortality in young adults. Understanding the T1D etiology will allow the design of preventive strategies to avoid or delay the T1D onset and to help to maintain control after developing. T1D development involves genetic and environmental factors, such as birth delivery mode, use of antibiotics, and diet. Gut microbiota could be the link between environmental factors, the development of autoimmunity, and T1D. In this review, we will focus on the dietary factor and its relationship with the gut microbiota in the complex process involved in autoimmunity and T1D. The molecular mechanisms involved will also be addressed, and finally, evidence-based strategies for potential primary and secondary prevention of T1D will be discussed.

Keywords: Type 1 diabetes; autoimmunity; diet; gut microbiota; dysbiosis; *Bacteroides*

1. Introduction

Type 1 diabetes (T1D) is one of the two most frequent autoimmune disorders in childhood and adolescence. It is due to the cellular-mediated autoimmune destruction of pancreatic β-cells, which leads to an absolute insulin deficiency, disturbing glucose metabolism [1]. The T1D prevalence of 1:300 is increasing over the world, representing 5%–10% of all diabetes mellitus cases [2].

Long-term micro- and macrovascular complications of diabetes are the leading causes of mortality [3] and disability in young adults. Understanding T1D etiology will allow for the design of preventive strategies to avoid or delay T1D onset and help to keep it under control if developed.

Genetic predisposition is the main determinant involved in T1D development, with the human leucocyte antigen (HLA) DR3-DQ2 and DR4-DQ8 haplotypes as the most common variants involved, which are shared with other autoimmune diseases such as celiac disease [4]. Since pancreatic β-cell autoimmunity appears frequently in the first 6 years of life, and its progression towards T1D can occur in preschoolers or during puberty, the factors investigated as possible triggers are related to early life and the immune system maturation process [5,6]. In addition to genetics, other factors such as birth delivery mode, diet, infections, and the use of antibiotics have been associated with T1D development [7]. However, the causality and possible mechanisms by which these factors relate to T1D remain unclear.

During the last decade, advances in molecular techniques have allowed for the study of gut microbiota in animal models of T1D [8] and, more recently, in children with autoimmunity and T1D [9–15]. The gut microbiota could be the link between environmental factors and the development of autoimmunity and T1D. This has led to the proposal of a possible intestinal origin of T1D [9], and has placed the microbiota as the central factor for its study.

In this review, we are focusing on the dietary element and its relationship with the gut microbiota in the complex process towards autoimmunity and the progression to T1D. The molecular mechanisms

involved will also be addressed, and evidence-based strategies for potential primary and secondary prevention of T1D will be discussed.

2. Diet and the Shaping of the Gut Microbiota

The first gut microbiota composition is mostly acquired at birth. The delivery mode determines the type of microorganisms that will colonize the newborn gut. Thus, children born vaginally develop a microbiota composed by *Lactobacillus*, *Prevotella* or *Sneathia* spp. from the maternal vaginal tract. Meanwhile, in those infants born by caesarean section, the bacterial communities from the mothers' or skin or the skin of participants in the surgical procedure, such as *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp., will dominate [10].

After delivery, diet is one of the main modulators of infant gut microbiota. Diet acts in a direct way by providing the substrates and sources of bacterial contamination from breast and nipple skin in breastfeeding babies or due to the tools and preparation methods in bottle-fed babies with infant formulas. Diet also contributes indirectly in the regulation of intestinal and pancreatic physiology [11]. During early childhood, microbiota diversity rapidly increases and new strains are acquired. Breastfeeding increases the diversity of lactic acid bacteria, while infant formulas contribute to the acquisition of bacterial communities such as *Staphylococcus aureus, Clostridium difficile, Bacteroides* spp., and other pathogenic communities. The microbiota structure is very unstable until the age of 2–3 years and it responds to changes in the diet, such as the introduction of solid foods or diseases; in subsequent years, it resembles the adult composition [12,13].

Around the age of 7 years old, the most prevalent phyla are *Firmicutes* and *Bacteroidetes*, representing about 90% of microorganisms, while the remaining 10% consists of *Proteobacteria*, *Tericutes* and *Cyanobacteria*. Three enterotypes have been proposed for the world population, in accordance with with the clustering patterns seen in the variations in the levels of the dominant microbiota genera: *Bacteroides, Prevotella,* and *Ruminococcus* [14]. In adults, these enterotypes have been associated with long-term dietary patterns. Thus, the *Bacteroides* enterotype has been correlated with diets dominated by high levels of animal protein and saturated fats, as occurs in the western diet. On the other hand, the *Prevotella* enterotype is more prevalent in people with higher consumption of carbohydrates and simple sugars, as observed in agrarian and vegetarian societies [15]. These enterotypes appear to be stable in adults after 6 months despite changes in saturated fats and fiber in feeding patterns [16].

In the last 5 years, several studies have examined the microbiota of healthy school-age children from different regions around the world. In all cases, the age, dietary patterns, and geography/traditions were the main determinants explaining the differences in gut microbiota composition. For example, microbiota profiles rich in *Prevotella* have been described in children from Burkina Faso [17], Mexico [18], Indonesia [19], Thailand [19,20], Malawi [21], and Amerindians from the Venezuelan Amazon [21]. All of them have common diets with a low content of fat and animal protein, and a high content of starch, fiber, and plant polysaccharides. In contrast, in the same age group of the United States [21], Italy [17], China [19], Japan [19] and Taiwan [19], the present gut microbiota is dominated by *Bacteroides*. In these, the diet is westernized with a high content of animal protein and fat, and a low fiber content.

Recently, the enterotype hypothesis has been questioned and reformulated because, according to Knights *et al.* [22], the stability of microbiotal composition could arise because "people resemble themselves over time in general rather than because there are specific barriers to switching cluster types". They demonstrated a temporal fluidity of enterotypes in a gradient form in which one individual can move across time. Hence, enterotypes can be unstable, continuous, and driven by sampling frame. Therefore, for a better understanding of the way that diet shapes the microbiota and in order to minimize bias, multiple sampling is recommended to avoid isolated "snapshots" of a dynamic process. It would also be useful to know the microbiotal composition throughout the entire gastrointestinal tract; however, this requires invasive techniques that are impractical in healthy people [23].

3. The Immunity-Diet-Microbiome Consortium: Towards T1D in Early Life

The modulation of the immune system by the microbiota begins even before birth. The intrauterine environment of the fetus during pregnancy is not completely sterile. There is evidence that the placenta of a term pregnancy has a non-pathogenic commensal microbiota in low-abundance, similar to the oral microbiome of non-pregnant women [24]. This suggests that from a very early stage, the fetus is exposed to bacterial antigens against which it has to develop tolerance.

The intestinal immune system begins to develop after 11 weeks of gestation. At 16 weeks, there are already functional B and T cells. However, the response to antigens remains blocked to protect the fetus from an overreaction of the immature immune system. This is possible because the amniotic fluid contains endotoxin-neutralizing histones and a lipopolysaccharide-binding protein that prevents the activation of the Toll-like receptor (TLR) pathway [25].

After birth, diet and microbiota are the decisive factors that guide the proper maturation of the immune system. Diet is a source of nutrients, but it is also the main route of entry for antigens to the organism [26]. At the same time, early colonizer microbiota produce stimuli that manage the differentiation of cells and tissues of the immune system [25]. At this stage, the infant immune system learns to distinguish the self from the non-self and to control the balance between regulatory and inflammatory responses in the host, due to the types of bacteria that form the gut microbiota [27].

To accomplish this, the immune system applies two adaptive anti-inflammatory strategies: first, the production of secretory IgA (sIgA) to prevent epithelial penetration and to control colonization over the surface towards the lumen. The second strategy is the development of oral tolerance, which helps to prevent hypersensitivity reactions against innocuous antigens that pass through the intestinal barrier [28].

A mutualistic relationship with the microbiota can occur because the gut epithelial cells express microbe-associated molecular pattern (MAMP) receptors, primarily TLR. The NF-κB pathway is activated by TLRs, producing a pro-inflammatory response. This results in the production of cytokines, chemokines, and antibacterial products, according to the type of TLRs that are activated and the microbiotal patterns that are being recognized. For example, the bacterial lipopolysaccharide (LPS) inhibits the interleukin-1 receptor-associated kinase (IRAK) M, a modulator of IRAK1, which is necessary for NF-κB activation. Similarly, the ubiquitination and degradation of IγB is inhibited by reactive oxygen species (ROS) which is induced by the microbiota and, peroxisome proliferator-activated receptor gamma (PPARγ), a product of the activation of Toll-like recepetor (TLR) 4 by LPS, diverts NF-κB from the cell core [29].

In this process initiated by bacterial recognition, there is also production of sIgA, differentiation of effector T helper (Th) 1, Th2, and Th17 cells, and the development of regulatory T cells (Treg). The differentiation of Tregs can be induced by commensal microbiota in the colon, such as Cluster IV and XIVa *Clostridia*, related to their short-chain fatty acid production, which stimulates the expression of Foxp3 in CD4+ T cells [27]. However, other bacterial communities can induce the production of inflammatory T cells. In this setting, the segmented filamentous bacteria can colonize the gut by getting in direct contact with the epithelium, facilitating their presentation by dendritic cells (DCs). This elicits a specific effector host response, characterized by a cascade of pro-inflammatory signals that culminate with the production of Th17 and Th1 cells, mediated by interleukin (IL)-1, IL-6 and IL-12, which can lead to autoimmunity [29]. Thereby, alterations in this process, such as dysbiosis or an inadequate introduction of foods during the first months of life, may increase susceptibility to and generate the development of autoimmune diseases, allergies and other disorders, locally in the gut or at a systemic level.

Besides this, the microorganisms of the microbiota can regulate the intestinal architecture, altering gut permeability. Epithelial cells are bound within each other by structural proteins such as zonulin, claudin, occluding, and actin [30]. For instance, enteropathogenic *E. coli* acts directly on the distribution of occludin and *Clostridium difficile* through its toxins A and B, can disorganize actin and dissociate the zonulin complex, increasing permeability by a paracellular route. Moreover, *Vibrio cholerae* produces

the zonula occludens toxin, which is homologous and competes with zonulin causing loss of the tight junctions [31]. As a result, dietary antigens and microbiotal products can pass through the leaky gut and initiate the development of an autoimmune response in genetically predisposed individuals.

Dietary antigens associated with T1D depend on early feeding regimens, the age of introduction of foods, especially wheat, to the infant's diet, and the current consumption of nutrients [26]. In contrast, breastfeeding has beneficial immunomodulatory effects in the newborn. Studies in mice have confirmed that passive-transferred sIgA prevents the translocation of bacteria in the intestine, promoting gut homeostasis, which protects against infection by pathogens [32]. In contrast, milk formula consumption has been historically associated with T1D.

In Finnish children from the Diabetes Prediction and Prevention (DIPP) study [33] and in Americans from the Diabetes Autoimmunity Study in the Young (DAISY) study [34], it has been found that fat intake from bovine milk products as well as proteins from fresh milk presented an increase in the risk of advanced β-cell autoimmunity and subsequent progression to T1D. The presence of high titers of anti-β-casein at diagnosis of patients with T1D and with latent autoimmune diabetes of adults (LADA) has been shown [35]. The A1, A2, and B variants of the bovine β-casein contain the PGPIP (Pro-Gly-Pro-Ile-Pro) motif repeated several times in their sequence. This motif is also repeated in the glucose transporter GLUT2, present in the pancreas. Therefore, a possible explanation for pancreatic damage is a cross-reaction of the immune system initially directed against a dietary antigen. Meanwhile, in the sequence of human β-casein, proline is replaced with valine, avoiding the immunogenicity against the human protein [26].

In addition, it has been proposed that high-gluten diets could be one of the primary drivers for gut dysbiosis associated with the T1D development [36]. This is related with the timing and amounts of dietary gluten fed to infants. The progressive introduction of gluten-containing foods to the diet, in terms of quantity, between 3 and 7 months after birth, can decrease the risk of T1D-associated autoimmunity [37]. T1D children have an altered T-cell reactivity to wheat antigens in the gut and peripheral tissues [9]. Recently, we found that 96% and 20% of the studied T1D Mexican children presented high titers of IgG isotypes anti-gliadins and IgA anti-gliadins, respectively [38]. This was an expected finding since T1D shares its genetic HLA-associated risk with celiac disease.

The effects of gluten on intestinal homeostasis are multiple. Gluten increases gut permeability, affecting the tight junctions, which is well described in celiac disease patients and recently in T1D. As a result, long gliadin peptides can move between the epithelial cells to the lamina propria. Then, the dendritic cells can detect them and migrate to other sites, such as the pancreatic lymph nodes, to activate autoreactive T cells [37]. In an *in vitro* study by Hamari *et al.* [39], it was found that pre-T1D children with multiple autoantibodies and those newly diagnosed with T1D presented a T-cell response against gliadin in a lower frequency and intensity than healthy controls and patients with long evolution T1D. This finding supports the idea of an aberrant immune response related to the development of T1D.

Considering all the results together, the interplay between diet, microbiota, and immune system could partly explain the origin of T1D in susceptible children. These mechanisms reflects the immune link between the pancreas and intestine, as they both develop from the embryonic endoderm [9].

4. The Diabetogenic Microbiome

Over the past decade, the first microbiotal studies in animal models suggested the novel possibility of T1D prevention in humans through gut microbiota modulation. Among them, the experiment by Brugman *et al.* [40], performed in Bio-Breeding diabetes prone (BB-DP) rats, proved that antibiotic treatment had an effect on the incidence of diabetes, and that the differences in gut bacterial composition were detectable before the rats developed disease. In another work by Wen *et al.* [8], the interaction between intestinal microbiota and the innate immune system was recognized as an epigenetic factor which can modify predisposition to T1D. In their study, specific-pathogen-free (SPF) non-obese diabetic (NOD) mice lacking myeloid differentiation primary response 88 (MyD88) did

not develop T1D. The MyD88 protein is an adaptor for TLRs and other innate immune receptors that recognize microbial stimuli. Furthermore, they found that this effect was related to commensal microbiota, as germ-free MyD88-negative NOD mice developed diabetes.

More recently, the antibiotic effects over the development of T1D has been further analyzed. It has been found that both the administration of broad spectrum antibiotics, which almost completely eliminate the commensal microbiota, and the use of selective antibiotics, which affect the microbiotal composition and limit certain bacterial groups, increase the incidence of T1D in NOD mice [41]. Moreover, fecal transplant from NOD to non-obese resistant (NOR) mice produced insulitis in the latter, and antibiotics accelerated the appearance of T1D in this model [42]. These studies suggest that antibiotics could potentiate the diabetogenic effects of the altered microbiome.

Research in this topic has strongly increased in the last five years. Taking advantage of the new high-throughput sequencing techniques and bioinformatics, studies in humans have been conducted, looking for the possibility of a diabetogenic microbiome. In Table 1, a comparison of the published studies to date is shown. It is remarkable that in all of these studies, the presence of dysbiosis has been related to the autoimmune process and its further progression to T1D. In Finnish patients, this imbalance has been associated with a decreased bacterial diversity after seroconversion, before the onset of hyperglycemia [43–45]. The development of β-cell autoimmunity may precede the appearance of hyperglycemia for over 15 years [5]. This represents a window of opportunity for possible microbiota-related therapies that could prevent or delay the development of T1D in autoimmune-presenting children.

The pattern of relative abundance of gut bacteria is different among the conducted studies. However, regardless of ethnicity, age and geography, all studies have detected *Bacteroides* as the main genus leading to T1D-associated dysbiosis. An increased proportion of *Bacteroides* in White Americans [46] and Caucasian children [9–11,13] with beta-cell autoimmunity, as well at the onset of T1D of Mestizo children [18], has been reported. Furthermore, there is a directly proportional relationship between the number of T1D-associated autoantibodies and the abundance of *Bacteroides* [44,45]. Hence, the presence of a higher degree of dysbiosis could contribute to the fast progression towards T1D that occurs in children with multiple autoantibodies [5].

An interesting finding of Davis-Richardson *et al.* [47] was the identification of two specific species causing the increase in gut abundance of *Bacteroides*: *B. dorei and B. vulgatus,* with *B. dorei* significantly increased before seroconversion. Therefore, the authors proposed to use them as predictors of T1D-associated autoimmunity. However, no other studies exist that confirm the increased presence of these species in other populations or their power as a predictor tool for T1D. In addition, we found that T1D Mexican children [18] with more than 2 years of evolution, controlled with insulin, presented a lower *Bacteroides* abundance than children with T1D at onset. These patients also had a relatively increased abundance of *Prevotella*, approaching the microbiotal profile described for healthy children with the same age and population.

Possibly, the full rebalancing of the proportion *Bacteroides* to *Prevotella* was not reached in our study [18] due to the diet of patients with T1D. The American Diabetes Association (ADA) [1] recommends an intensive insulin therapy scheme, using the carbohydrate counting method. This allows the patients to have a close to "normal" diet according to their customs. However, to achieve the goals of glycemic control, it tends to limit the dietary load and glycemic index, reducing carbohydrate intake and increasing fat consumption compared to healthy children. In the study by Virtanen *et al.* [48], 38 Finnish children with T1D were followed to analyze their diet. The proportion of energy from fats increased in these children from 26% at onset of disease to 30% two years later. The American Heart Association recommends limiting the intake of saturated fat to 7% of energy to prevent cardiovascular events [49]. In the same study [48], they found that most of the fat sources consumed by T1D children were of animal origin and the saturated fat consumption was around 11%, high above the recommended level. This suggests that the high fat diet may be maintaining the *Bacteroides* levels, limiting the full recovery of the microbiotal balance.

Table 1. Comparison of microbiota composition in humans with autoimmunity and T1D.

Country/Ethnicity	Diagnostic (*n*)	Age in Years	Microbiota Diversity in Autoimmunity/T1D	Microbiota Relative Abundance in Autoimmunity/T1D	Other Findings
Finland (DIPP Study)/Caucasians [43,50]	β-cell AI (4) HC (4)	0–2 0–3	Reduced	F/B ratio ↓ Increase in: ***Bacteroides*** genus, mainly *Bacteroides ovatus.* Decrease in: *Prevotella and Faecalibacterium*	Non-butyrate producers avoid optimal mucine synthesis in T1D-associated autoimmunity.
Spain/Caucasians [51]	T1D at onset (16) HC (16)	7.16 ± 0.72 7.48 ± 0.87	Similar to the control group ($p > 0.05$)	F/B ratio ↓ Increase in: *Clostridium*, ***Bacteroides*** *and Veionella*. Decrease in: *Lactobacillus*, *Bifidobacterium* and *Prevotella.*	Microbiota differences were associated with glycemic level.
Finland (FINDIA and TRIGR studies)/Caucasians [44]	β-cell AI (18) HC (18)	FINDIA/TRIGR: $5.1 \pm 1/13.3 \pm 1$ FINDIA/TRIGR: $5.0 \pm 2/12.8 \pm 1$	Reduced	F/B ratio ↓ Increase in: ***Bacteroides*** genus, *Clostridium perfringens.* Decrease in: *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum.*	The abundance of lactate- and butyrate-producing bacteria was inversely related to the number of β cell autoantibodies.
Mexico/Mestizos [18]	T1D at onset (8) T1D $\geq$ 2 years evolution (13) HC (8)	12.3 ± 0.64 11 ± 1.04	Similar to the control group ($p > 0.05$)	Unaltered F/B ratio. Increase in: ***Bacteroides*** genus. Decrease in: *Prevotella, Acidaminococcus* and *Megamonas.*	The glycemic control in the T1D $\geq$ 2 years treated group partially normalizes the microbiotal profile towards *Prevotella*-dominant profile.
Finland (DIPP Study)/Caucasians [47]	High risk cohort (76): β-cell AI (29) T1D at onset (22) HC (47)	0–2	Reduced	F/B ratio ↓ Increase: in ***Bacteroides*** genus due to *Bacteroides dorei* and *Bacteroides vulgatus.*	*B.dorei* abundance peaked over 8 months prior to the appearance of the first islet auto antibody. It coincided with the introduction of solid foods.
Finland, Estonia (DIABIMMUNE Study)/Caucasians [45]	High risk cohort (33): β-cell AI (7) T1D at onset (4) HC (22)	0–3	Reduced	Increase in: *Blautia, Rikenellaceae, Ruminococcus gnavus* and *Streptococcus infantarius* in T1D cases at the time of alpha-diversity divergence.	Decreased community diversity occurs after seroconversion but before onset of T1D. T1D onset is preceded by increased inflammation-assoc. organisms and pathways.
USA/White Americans (TRIALNET Study) [46]	β-cell AI (21) T1D at onset (35) Seroneg. FDR (32) HC (23)	4–49 2–20 3–45 4–24	Similar to the control group ($p > 0.05$)	Increase in: ***Bacteroides.*** Decrease in: *Prevotella* * In seropositive subjects with multiple *versus* one autoantibody.	The microbiomes of β-cell AI and seroneg. FDRs clustered together but separate from those of T1D at onset and HC.

T1D: Type-1 Diabetes; β-cell AI: β-cell autoimmunity; F/B ratio: Firmicutes/Bacteroidetes ratio; HC: Healthy controls; FDR: First degree relatives. DIPP: Diabetes Prediction and Prevention; FINDIA: Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes; TRIGR: Trial to Reduce Type 1 Diabetes in the Genetically at Risk; TRIALNET: Type 1 Diabetes TrialNet; * indicate that these findings (Increase in: *Bacteroides*, Decrease in: *Prevotella)* were only detected in seropositive subjects from the TRIALNET study with multiple versus one autoantibody and there were not considered the seronegative first degree relatives included in the original study design.

5. Microbiota: Molecular Mechanisms in T1D

To explain the pathways and the impact of T1D-associated dysbiosis in the metabolism, it is necessary to study the microbiota structural dynamics as an integral organ [52]. Understanding that the gut microbiota is an organ will make it possible to integrate its relationship with T1D as a key for designing new therapies to prevent and/or improve the T1D control.

Dietary components provide different substrates that may result in several products during the fermentation processes. Changes in the microbiotal structure due to diet modifications are because some of the bacterial communities are "genetically better equipped" to metabolize those substrates. Moreover, the same substrate can be used in different pathways according to the type of bacteria that are colonizing the intestinal niche, or in relation to its abundance and available frequency [13]. An example of the former statement is lactate. This substrate can be transformed into butyrate or, in others, short chain fatty acids (SCFA) such as acetate, succinate, and propionate during its anaerobic bacterial fermentation in the gut, depending on the type of microbiota [50].

The lactate model appears to be the strongest possible explanation for understanding the link between T1D and dysbiosis (Figure 1). According to this model, the presence of lactic acid- and butyrate-producing bacteria such as *Prevotella* and *Akkermansia* helps to maintain a healthy epithelium. This is because butyrate contributes to mucin synthesis and to the assembly of tight junctions [53]. These bacteria were common in the microbiota of healthy children around the world [9–12,15,50]. In contrast, when microorganisms such as *Bacteroides* and *Veillonella* are harbored in abundance, this substrate follows the pathway to succinate, acetate, and propionate. These products compromise mucin synthesis and increase paracellular permeability by altering the tight junctions [50].

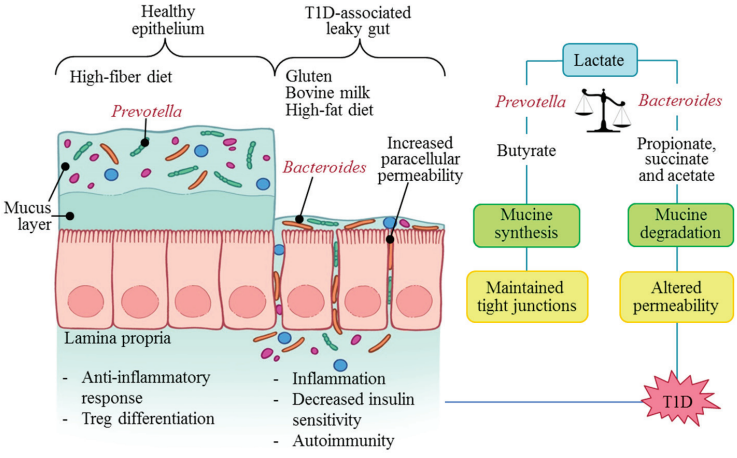

Figure 1. Diet and microbiota associated mechanisms in autoimmunity and type 1 diabetes (T1D) development.

In addition, butyrate may contribute to maintaining the anti-inflammatory response in the healthy gut by inhibiting the activation of NF-κB, signaling through G protein-coupled receptors, and leading to the modulation of antioxidant defense systems, nitric oxide production, and the expression of inflammatory cytokines [28,36]. High-fiber diets have been associated with a decreased risk of inflammatory immune-related diseases. However, it is unknown whether this effect is due to the butyrate itself or to the associated microbial profile [28]. Butyrate also enhances extra-thymic differentiation of Treg cells, while other SCFA, such as acetate, block this process [54]. Treg differentiation seems to be related to histone acetylation in the promoter of the Foxp3 locus, which is also regulated by butyrate [36]. This suggests that microbiota-derived products function as mediators in

the communication between bacteria and the host immune system, leading to pro- or anti-inflammatory responses [54], and could be a factor involved in β-cell autoimmunity and T1D.

Systemic effects of intestinal butyrate in the regulatory immune response also occur at the pancreatic level. Butyrate has been associated with the expression of cathelicidin-related antimicrobial peptide (CRAMP) in the β-cells of NOD mice. This peptide has been shown to protect against the development of T1D by inducing a regulatory response and suppressing the inflammatory process in the pancreatic islets of prediabetic mice [55].

Free fatty acid receptor 2 (FFAR2) is one of the G protein-coupled receptors that can be activated by microbiota-derived butyrate. This receptor is involved in the insulin signaling regulation in adipose tissue and in the maintenance of energy homeostasis. Its activation promotes the secretion of GLP-1 in the intestine, the suppression of fat accumulation, and, therefore, increased sensitivity to insulin [56]. This is an interesting mechanism because even though the main problem in T1D is not insulin resistance, the diabetes accelerator theory puts it in context. This theory proposes that in T1D, body constitution, insulin resistance, and autoimmunity are three processes that accelerate the loss of beta-cells by apoptosis [57]. This theory arises when observing that children with autoimmunity that progress more quickly to T1D have greater insulin resistance than those non-progressors [58]. Thus, decreased production of butyrate in children with low levels of *Prevotella* in their gut microbiota could be contributing to T1D development.

Kostic *et al.* [45], in the DIABIMMUNE study, followed children with high genetic risk for T1D from the first days following birth. Their results show associations between the gut bacterial communities and metabolic profile in young children, such as the levels of *Blautia* with long-chain triglycerides and *Ruminococcus* with short-chain triglycerides. Furthermore, these two microorganisms, which were abundant in children who progress to T1D, correlated positively with the presence of branched-chain amino acids such as valine, isoleucine, and leucine. Meanwhile, Oresic *et al.* [59] found that the dysregulation of lipid and amino acid metabolism precedes the appearance of glutamic acid decarboxylase and insulin autoantibodies in children who later developed T1D.

6. T1D Prevention and Control Possibilities

Children born with a genetic risk of T1D represent 30% of all births, but most of them do not develop the disease [60]. The risk increases considerably when β-cell autoimmunity appeared; according to the TEDDY study group [61], this can be attributed to the presence of two or more associated autoantibodies. With the appearance of autoantibodies, the risk for T1D increases up to 75% in the next 10 years and it is almost certain within 20 years [62,63]. Therefore, it is essential to implement effective prevention strategies at three levels of attention: primary prevention, before seroconversion; secondary prevention, when the β-cell autoimmunity is already present, trying to prevent or delay the T1D onset in predisposed children; and tertiary prevention, when the T1D is already present, to avoid complications [62].

6.1. Primary Prevention of T1D

Based on the evidence, the primary prevention of T1D should focus on modifiable perinatal factors, which theoretically could help to prevent not only T1D, but other autoimmune and allergic diseases in children. Thus, as the newborn initial microbiota is primarily obtained from the mother during birth and lactation, the possibility of considering maternal microbiome as the starting point has been suggested [52]. Thus, maintaining a healthy maternal microbiota, avoiding unnecessary cesareans, and the promotion of breastfeeding are important activities in which both mothers and health caregivers have to be educated.

Dominguez-Bello *et al.* [64] evaluated the possibility of modulating the gut microbiota from cesarean-born children. The newborns were exposed to a first natural inoculum, obtained from the *Lactobacillus*-dominated vaginas of their healthy mothers, in order to mimic the probable microbiota that they would have acquired if they were born vaginally. Preliminary results show that these babies

have bacterial communities with an intermediate pattern between children born vaginally and those born by cesarean not receiving the inoculum. Moreover, regarding lactation, breastfeeding must be encouraged. It is also important that mothers have a varied and balanced diet while nursing. It was recently found that maternal consumption of red meats, meat products, and vegetable oils increases the risk of the baby developing autoimmunity and T1D in the following years [65]. In turn, in those who are exclusively milk formula-fed, supplementation with prebiotics and probiotics have proven to be effective in modifying the intestinal microbiota, resembling the profile of infants who are breastfed, stimulating the proper maturation and function of the immune system [11]. However, the effectiveness of these practices long-term has not yet been proven and it is not known whether they are sufficient to counteract the negative effects associated with the consumption of cow's milk proteins in early life.

Regarding bovine milk proteins contained in milk formulas, an option to prevent the development of autoimmunity in children at high risk might be weaning with a highly casein-hydrolyzed formula. To confirm this, the TRIGR study [66] is evaluating whether or not the use of hydrolyzed formula is safer than conventional milk formulas. The final endpoint of this study is in 2017, when participants turn 10 years old. This is a study with enough power to confirm or reject this theory and to provide the certainty required to direct preventive strategies. The possible benefits of highly hydrolyzed formulas in reducing the risk of autoimmunity with respect to conventional formulas are wide. Among them, they may avoid early exposure to intact bovine insulin, decrease gut permeability and thus the paracellular transit of foreign peptides, induce maturation of Tregs, decrease inflammation, and, with this, potentially contribute to maintaining the diversity of the intestinal microbiota.

6.2. Secondary and Tertiary Prevention of T1D

Once the autoimmune process has started, diet is the main known modifiable factor capable of changing the risk of developing T1D. The progression to T1D in children with β-cell autoimmunity is associated with the intake of total sugars, especially from sugar-sweetened beverages in those with a high-risk genotype [67]. The results from the latest clinical and experimental studies suggest that an effective measure in diabetes could be to target the treatment to the modulation of microbiota [68]. Considering the current information, dietary interventions should focus on having a greater impact on the metabolic function of the microbiota rather than on its composition [23]. Still, much remains to be understood about T1D etiology. In other diseases which also have an inflammatory gut background, the use of probiotics and prebiotics has been tested for their management. However, despite these strategies enabling the increase in the abundance of specific bacteria at the genus and species levels, changes in the overall composition of the microbiota are small and are kept only during the intervention period [69].

Other possible practices for prevention and/or treatment include fecal transplantation and the use of mucosa-protective drugs to manage leaky gut syndrome. Although fecal transplant is the only way to completely change microbiome, it is still unknown how unstable the new ecosystem could be, and therefore, the duration and efficacy of its effect in pre-T1D patients. It must also be considered that if fecal transplant would be performed without any dietary intervention, its effect, if significant, would be short-term. In T1D patients, fecal transplant accompanied by diet intervention could help achieve glucose control and recover microbiotal balance. Regarding mucosa-protective drugs, there are new drugs, such as gelatine tannate, that could be used as an intestinal barrier-modulating drug. According to the first trials, this drug may favor a physiological permeability, creating a bioprotective film by forming bonds with mucin, avoiding the aggressive penetration of bacteria, restoring functionality, and thus inducing an indirect anti-inflammatory effect [70].

Finally, in order to treat patients who do not respond adequately to conventional treatment, and investigating the possibility of remission and/or cure of the disease, other therapeutic strategies for T1D are pancreas transplant, pancreatic islet transplant , and, more recently, stem cell therapy [71]. Stem cell therapy seeks to take advantage of the regenerative capacity and immunomodulatory effects of the pluripotent cells. However, none of them have been effective in clinical practice alone in the long

term. According to Chhabra and Brayman [72], safe stem cell strategies should be combined with other techniques, such as islet transplant, using the latest gene therapies and novel immunosuppressive and immunomodulatory drugs. In addition, the modulation of intestinal microbiota through fecal transplant and dietary intervention may help maintain the beneficial effects of the discussed techniques long term.

7. Conclusions

The composition of the gut microbiota can be modulated by diet. This modulation can promote the proper maturation of the immune system, or, result in gut dysbiosis and aberrant immune responses that can lead to autoimmunity and T1D in predisposed children. Thus, dietary antigens and microbiota-derived products could be acting as triggers of T1D by promoting a pro-inflammatory and metabolic dysfunctional environment. The genus *Bacteroides* is the largest representative of T1D-associated dysbiosis. Among the possible strategies for prevention and treatment, fecal transplant accompanied by dietary intervention appears to be the most promising option for the prevention of T1D in children with autoimmunity.

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Review

The Intestinal Microbiota in Metabolic Disease

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Abstract: Gut bacteria exert beneficial and harmful effects in metabolic diseases as deduced from the comparison of germfree and conventional mice and from fecal transplantation studies. Compositional microbial changes in diseased subjects have been linked to adiposity, type 2 diabetes and dyslipidemia. Promotion of an increased expression of intestinal nutrient transporters or a modified lipid and bile acid metabolism by the intestinal microbiota could result in an increased nutrient absorption by the host. The degradation of dietary fiber and the subsequent fermentation of monosaccharides to short-chain fatty acids (SCFA) is one of the most controversially discussed mechanisms of how gut bacteria impact host physiology. Fibers reduce the energy density of the diet, and the resulting SCFA promote intestinal gluconeogenesis, incretin formation and subsequently satiety. However, SCFA also deliver energy to the host and support liponeogenesis. Thus far, there is little knowledge on bacterial species that promote or prevent metabolic disease. *Clostridium ramosum* and *Enterococcus cloacae* were demonstrated to promote obesity in gnotobiotic mouse models, whereas bifidobacteria and *Akkermansia muciniphila* were associated with favorable phenotypes in conventional mice, especially when oligofructose was fed. How diet modulates the gut microbiota towards a beneficial or harmful composition needs further research. Gnotobiotic animals are a valuable tool to elucidate mechanisms underlying diet–host–microbe interactions.

Keywords: intestinal microbiota; obesity; diabetes; metabolic syndrome; energy harvest; diet; absorption; bile acids; low-grade inflammation; SCFA

1. Introduction

Recent years have seen a surge in publications reporting correlations between the gut microbiota and various medical conditions such as inflammatory bowel disease, colorectal cancer, allergies, autism and kidney stones. This development has been fostered by considerable technological progress and the advent of the omics technologies, which afford a fast and relatively inexpensive culture-independent assessment of complex microbial communities, their gene repertoire (the microbiome), gene expression and metabolic profiles. The role of intestinal bacteria in the development of obesity and associated diseases has attracted particular attention, not only in the scientific community but also in the lay press, because it has become a major public health issue. Even though obesity and metabolic disease are considered to be nutrition-related disorders, recent evidence indicates that the intestinal microbiota plays a major role in disease development.

2. Intestinal Microbiota

The gut microbiota of a given animal species has co-evolved with its host such that it is optimally adapted to the intestinal environment of the respective host. Thus, it is not surprising that the microbial community inhabiting the digestive tract affects host physiology in many ways, mainly by interacting with the host immune system and by broadening the metabolic potential of the host. The majority of microorganisms in the gut are considered commensals, as they perform tasks that are beneficial for

the host. However, even though this microbial community usually lives in harmony with its host, it should be kept in mind that gut bacteria are not altruistic but solely taking advantage of the constant temperature and the wide range of substrates available in the digestive tract. In return, by virtue of its immense metabolic potential, the intestinal microbiota makes otherwise non-utilizable nutrients available to the host. For example, non-digestible carbohydrates, also referred to as dietary fiber, are fermented to short-chain fatty acids (SCFA), which can be utilized by the host. However, under certain circumstances, the harmonic relationship between the host and its microbiota gets lost. Possible reasons include medication, a diseased state and/or unhealthy nutrition. Interestingly, various diseases are accompanied by alterations in the gut microbiota, often referred to as dysbiosis.

2.1. Composition

The gut microbiota of adult healthy subjects is dominated by six bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia. Besides these phyla of the domain Bacteria, the human digestive tract also harbors the methanogen *Methanobrevibacter smithii* (*M. smithii*), which belongs to the Archaea domain and can be found in every second individual, as well as eukaryotic organisms, such as the yeast *Candida*. While methanogens and yeasts contribute to less than 1% of all microbial cells of the fecal microbiota, Firmicutes and Bacteroidetes together can reach a proportion of more than 90%, while the proportion of representatives of the other phyla ranges from 2% to 10% [1]. A comparison of data from various mouse studies indicates that the proportions reported for the different phyla differ quite considerably among these studies. For example, while the proportion of Actinobacteria in the study by Hildebrandt *et al.* was less than 1% [2], Murphy *et al.* reported Actinobacteria to make up 11%–25% [3]. These inter-study discrepancies are probably to a large extent due to differences in the protocols used for sampling, storage and DNA extraction. Indeed, a recent study demonstrated that the DNA extraction method is critical for the detection of clostridial and actinobacterial populations [4]. Therefore, protocols have to be rigorously tested and harmonized to make studies more comparable.

Most knowledge about the composition of the human gut microbiota stems from the analysis of fecal samples. However, it has to be kept in mind that microbiological data based on such analyses are not representative of the situation in the various gut sections. Moreover, there are considerable differences between the microbial communities in the gut lumen and those adhering to the mucus layer covering the intestinal mucosa [5].

2.2. Key Activities of the Gut Microbiota

Substrate availability and physicochemical conditions in the intestinal tract are key factors that affect the composition and activity of the gut microbiota. The majority of substrates required by intestinal bacteria for their growth stems from the diet. In addition, the host provides mucins, desquamated epithelial cells and digestive enzymes as additional substrates to intestinal bacteria. Important dietary substrates for gut bacteria include undigested or incompletely digested carbohydrates such as resistant starch and dietary fiber. The latter includes a large variety of carbohydrate polymers typically found in dietary plants. Cellulose, hemicellulose and pectin are components of the plant cell wall, whereas inulin is used by some plants for carbohydrate storage. In contrast to humans, who are devoid of enzymes capable of breaking down these carbohydrate polymers, the intestinal microbiome encodes a broad spectrum of enzymes that catalyze their depolymerization and further degradation. In fact, carbohydrate degradation pathways are over-represented in the human gut microbiome compared to other microbial genomes [6]. The underlying bacterial activities enable the host to take advantage of indigestible carbohydrates that otherwise would be excreted unused. The enzymes provided by the gut microbiome afford the depolymerization of a wide range of carbohydrates such as xylans, α- and β-glucans, fructans, β-mannans and pectins [7]. Intestinal bacteria involved in this process include members of both the Firmicutes (*Ruminococcus, Butyrivibrio* and *Roseburia* species) and the Bacteroidetes (*Bacteroides* spp.).

The starch utilization system (Sus) from *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) has been studied in detail [8,9] and been used as the paradigm for other polysaccharide degradation systems in *Bacteroides* spp. Much less is known about the carbohydrate utilization systems in members of the Firmicutes, even though these bacterial species play a major role in colonic carbohydrate fermentation [7,10].

Mucus produced by goblet cells represents another important source of growth substrates for intestinal bacteria. It can be utilized by a considerable number of intestinal bacteria, including *B. thetaiotaomicron* [11], *Bifidobacterium bifidum* [12], and *Akkermansia muciniphila* [13], a member of the phylum Verrucomicrobia. Intestinal mucins consist of up to 80% of glycans attached to a protein backbone, which accounts for approximately 20% of the molecule. *B. thetaiotaomicron* is one of the most versatile glycan utilizers in the intestine. However, this bacterium does not utilize various glycans simultaneously, but rather prioritizes their utilization, regardless of their dietary or host-derived origin [14]. This is accomplished by a highly sophisticated regulatory network, which involves hybrid two-component systems. The latter are transmembrane proteins consisting of two domains, which in classical bacterial two-component systems are made up of two separate proteins. The periplasmic domain of this transmembrane protein acts as a glycan sensor while the cytoplasmic domain contains a helix-turn-helix DNA-binding module that controls the expression of genes involved in glycan utilization [15,16].

Even though the majority of intestinal micro-organisms prefer glycans over proteins as growth substrates, proteins are also utilized, in particular in the distal colon where the availability of carbohydrates is limited because they have been used up in the proximal part of the intestinal tract [17]. The amount of dietary protein that reaches the colon is small but not negligible. In addition, digestive enzymes and desquamated epithelial cells are another protein source for colonic bacteria. Proteins reaching the colon are first cleaved into peptides and amino acids, which undergo further bacterial degradation. Bacterial amino acid degradation in the colon involves oxidative and reductive deamination reactions often followed by decarboxylations resulting primarily in the formation of SCFA. Further typical degradation products include amines, branched-chain fatty acids produced from iso-amino acids as well as phenolic and indolic compounds produced from aromatic amino acids [18–20].

Other activities of the intestinal microbiota include the conversion of secondary plant metabolites such as glucosinolates in Brassica vegetables [21,22] or polyphenols in fruits, vegetables, cereals, chocolate, tea, coffee, or wine [23]. Some transformation products formed by intestinal bacteria may have health-promoting properties and have therefore been a major topic in nutrition research.

Intestinal bacteria also play a role in the metabolism of xenobiotics and the conversion of bile acids. Xenobiotics are first oxidised and subsequently sulphated or glucuronidated to render them water soluble and thereby facilitate their urinary excretion. Following their synthesis in the liver, bile acids (cholic acid and chenodeoxycholic acid in humans) are conjugated with either glycine or taurine and then secreted into the intestinal tract, where they undergo deconjugation and partial dehydroxylation by intestinal bacteria [24].

In contrast, lipids cannot be utilized by anaerobic bacteria because the oxidation of long-chain fatty acids requires the presence of oxygen, which is scarce in the intestine. Therefore, changes in the gut microbiome observed in response to high-fat diets are rather due to changes in the intestinal environment. For example, a rat study revealed that oral administration of the bile acid cholate induced changes in the composition of the gut microbiota similar to those observed after feeding a high-fat diet [25], indicating that diet-related microbiota changes may be of indirect nature (see section 4 for more details).

3. Obesity and Metabolic Disease and Their Link to the Intestinal Microbiota

Obesity is often accompanied by dyslipidemia, hypertension and impaired glucose homeostasis, known as metabolic syndrome. The consumption of energy-dense foods as well as the low energy

requirement for physical activity and reproduction are the main determinants of obesity in Western countries. In the last decade the intestinal microbiota has been proposed as another environmental factor involved in the onset of obesity. However, to which extent and through which mechanism the intestinal microbiota contributes to obesity development has not yet been elucidated.

A comparison of germfree and conventional mice revealed that the intestinal microbiota contributes to an obese phenotype [26–29]. However, the conclusion that germfree C57BL/6 mice are generally protected from diet-induced obesity as reported by Backhed *et al.* (2007) could not be reproduced for all mouse strains and diets. Interestingly, germfree C3H mice were protected from obesity when fed the same Western diet used by Backhed and colleagues, whereas the feeding of a semi-synthetic high-fat diet with essentially the same macronutrient composition but other ingredients increased the body weight gain in these mice [30]. These and other discrepancies in the literature call for a better understanding of the interplay between diet and host health and for an elucidation of the exact role of gut bacteria in this interaction.

That the intestinal microbiota plays an important role in obesity development can be deduced from fecal transplantation experiments. Transplantation of fecal microbiota from obese mice to lean germfree mice also transferred the obese phenotype to the recipients. Mice that received the gut microbiota from lean mice stayed lean [31,32]. The adipose phenotype was even transmissible from human twins discordant for obesity to germfree mice by a single oral gavage of feces [33]. However, co-housing of mice that received the lean or the obese microbiota prevented increased adiposity in the recipients of the obese microbiota. Members of the Bacteroidetes present in the lean microbiota successfully invaded the obese microbiota when the mice were fed a low-fat, fiber-rich diet. This was accompanied by a reduction of body fat in the mice that originally received the microbiota from the obese donors. However, the invasion of Bacteroidetes and the prevention of adiposity failed when the mice consumed a high-fat, low-fiber diet [33]. This study demonstrates the close relationship between diet and intestinal microbiota since the microbial composition is modifiable by diet with direct consequences for host health.

4. Impact of Energy-Rich Diets on Microbiota

Dietary modifications change the intestinal microbiota of mice and humans within one day [32,34,35]. Such diet-induced changes in the gut microbiota are hypothesized to promote the development of obesity and associated chronic diseases. Diet selects for certain bacteria as shown by Ridaura *et al.* [33], but in which way dietary components exactly lead to the observed changes in the microbiota is largely unknown (Figure 1).

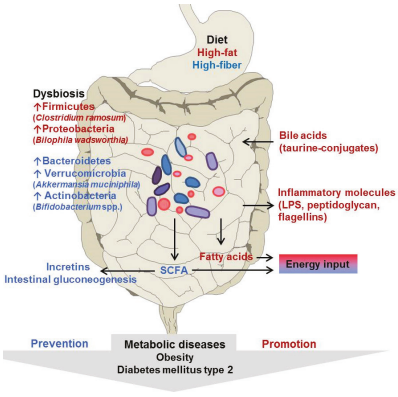

Figure 1. Hypothetical interplay between diet, gut microbiota and host in prevention and promotion of metabolic diseases. Consequences of high-fat diets and fiber-rich diets are indicated in red and in blue, respectively.

Dietary intervention studies in mice revealed an increase in Firmicutes and a decrease in Bacteroidetes in obese individuals [2,3,36]. These studies suggest that the observed microbiota changes in obese mice were caused by diet rather than by the obese phenotype. The high proportion of Firmicutes in obese mice fed high-fat diets was in part due to the proliferation of Erysipelotrichi, a bacterial class within this phylum, formerly known as Mollicutes [30,32,37]. However, genetically obese mice also harbor more Firmicutes and correspondingly less Bacteroidetes in their gut compared to their lean siblings [38], indicating that diet-independent host factors also modulate the microbiota.

In humans, obesity and the metabolic syndrome are also associated with a higher intestinal Firmicutes/Bacteroidetes ratio in comparison with lean or "healthy obese" individuals [39–41]. The consumption of calorie-restricted diets was accompanied by a reduction of body weight, and a shift from the high Firmicutes/Bacteroidetes ratio to a lower value typical of lean subjects [39]. Energy-rich diets were reported to increase the proportion of intestinal Firmicutes in both humans and mice, suggesting that dietary ingredients or endogenous metabolites secreted into the gut lumen in response to these diets (e.g., bile acids) were responsible for this phenomenon. However, other human trials not only failed to confirm a high proportion of Firmicutes in obese patients [42] but reported even the opposite [43]. The reasons for this discrepancy are not really known.

Amount and type of dietary fat affect the spectrum of bile acids formed in the liver and released into the intestine, which in turn influences the gut microbiota. For instance, a diet rich in saturated fatty acids promotes the hepatic production of taurine-conjugated bile acids at the expense of glycine-conjugated bile acids. The latter were dominant when an iso-caloric diet rich in polyunsaturated fat was fed [44]. Deconjugation of the taurine-conjugated bile acids stimulated the growth of *Bilophila wadsworthia* (*B. wadsworthia*) because this organism is able to gain sulfite from the taurine and to use it as an electron acceptor [44].

The administration of the primary bile acid cholic acid decreased the total bacterial cell count in the caecum of rats [25]. The authors proposed that microbial 7α-dehydroxylation of cholic acid to deoxycholic acid reduced the number of bacterial cells because deoxycholic acid has a 10 times higher bactericidal activity compared with cholic acid. Similar to the shifts at phylum level in response to high-fat feeding in mice [2,3,32,37], cholic acid-fed rats displayed an increase of caecal Firmicutes and a decrease of Bacteroidetes. The expansion of the Clostridia and Erysipelotrichi was mainly responsible for the high proportion of Firmicutes in these rats. The authors speculated that high dietary fat intake promoted the formation of deoxycholic acid, which in turn affected microbiota composition [25].

In humans who consumed an animal-based diet rich in dietary fat and protein, the gut microbiota was enriched with bile-tolerant taxa [35]. Similar to the changes in microbiota composition observed by Devkota *et al.* in mice in response to a diet rich in saturated fatty acids, the animal-based diet consumed by humans also promoted the expansion of *B. wadsworthia* in their intestinal microbiota [35]. It may be speculated that in both studies elevated intestinal bile acid concentrations promoted the outgrowth of this sulfite-reducing bacterium, which triggers colitis in genetically susceptible interleukin-10 knockout mice [44].

5. Influence of Intestinal Bacteria on Host Ability to Harvest Energy from the Diet

Bacterial degradation of non-digestible dietary polysaccharides to monosaccharides and their ensuing fermentation to SCFA by the intestinal microbiota is hypothesised to contribute to obesity development. A previous animal study indicated a link between high intestinal SCFA levels and obesity: Mice di-associated with *B. thetaiotaomicron* and *M. smithii* displayed higher caecal acetate concentrations, increased *de novo* lipogenesis and a higher epididymal white adipose tissue weight than germfree mice or mice mono-associated with either one of these strains [45]. Moreover, the metagenome of obese mice fed a Western diet was enriched in genes involved in the fermentation of simple sugars. Accordingly, the obese mice displayed elevated caecal SCFA concentrations [37]. In line with the notion that intestinal SCFA formation promotes overweight, a high-fat diet supplemented with a fermentable fiber fed to mice resulted in a higher body weight gain than the same high-fat diet except that the fermentable fiber had been replaced by a non-fermentable fiber [46]. This suggests that intestinal SCFA promoted body weight gain in the mice fed the fermentable fiber by delivering additional energy. In support of this explanation, obese mice fed high-fat diets poor in fermentable fiber displayed lower fecal acetate concentrations compared with lean mice fed a diet rich in fermentable fiber. However, the high acetate levels in the lean mice suggest that this lipogenic SCFA does not necessarily lead to obesity [30].

In humans, the consumption of diets rich in dietary fiber is associated with higher fecal SCFA concentrations as well as a lower incidence of obesity and symptoms of metabolic disease [47–49]. Therefore, dietary fiber is generally regarded as health conducive as it helps in the management of metabolic disease supposedly by virtue of the SCFA derived thereof by bacterial fermentation. However, human studies reported higher fecal SCFA levels in overweight and obese patients than in lean subjects, which was unlikely to be caused by a reduced colonic SCFA absorption or a higher intake of dietary fiber in the diseased subjects [43,50]. Similarly, in obese women who consumed an energy-dense diet, fecal SCFA concentrations correlated positively with adiposity and insulin resistance. However, the increased fecal SCFA levels could not be explained by the subjects' fiber consumption [51]. The discrepancies observed between reduced fiber intake and elevated fecal SCFA concentrations are difficult to reconcile and might have as yet unknown reasons. For example, it is conceivable that the role of intestinal SCFA in obesity development depends on dietary factors other than the amount of consumed fiber.

It is also worth mentioning that dietary fiber does not always change the dominant microbial groups and the concentrations of SCFA in the gut [52]. Hence, fibers may exert beneficial effects on the host independently from the microbiota and from SCFA formation. This may in part be due to the fact that dietary fiber decreases the energy density of food and/or impairs absorption of amino acids and small peptides in the upper digestive tract [53]. Subsequently, the diminished amino acid supply could prevent the amino acid induced increase in expression of ribosomal protein S6 kinase beta-1 in subcutaneous adipose tissue that would otherwise have promoted insulin resistance. Nonetheless, the beneficial effects of dietary fiber may at least in part be mediated by SCFA as they have been demonstrated to modify host energy metabolism (see section 8 for more details).

6. Influence of Intestinal Bacteria on Monosaccharide Absorption

The human diet is rich in carbohydrates including complex polysaccharides, disaccharides and monosaccharides. Simple sugars such as glucose and fructose are rapidly absorbed, while disaccharides such as maltose, sucrose and lactose have to be hydrolyzed to monosaccharides prior to absorption in the small intestine. Polysaccharides such as hemicellulose, pectin and resistant starch escape digestion in the upper digestive tract. In the ileum, processing of these polysaccharides by bacterial glycosidases delivers monosaccharides, which may contribute to the energy demand of the host. A comparison of conventionalized mice and germfree mice revealed that the presence of a microbiota improves intestinal glucose absorption [26]. Conventionalized mice displayed increased levels of serum glucose and serum insulin, both of which are known activators of the transcription factors carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein 1 (SREBP1), respectively. Activation of the acetyl-CoA carboxylase gene (*Acac1*) and the fatty acid synthase gene (*Fasn*) by these transcription factors was proposed to promote hepatic *de novo* lipogenesis in the conventionalized mice. However, which bacterial species mediated the increased intestinal glucose absorption was not reported.

A possible mechanism how gut bacteria facilitate glucose uptake might be an increased expression of glucose transporters. In support of this explanation association of germfree mice with *B. thetaiotaomicron* increased the ileal transcription of the sodium/glucose co-transporter 1 (*Slc5a1*). This transporter mediates glucose and galactose uptake in symport with sodium ions into enterocytes [54]. Interestingly, the presence of *Clostridium ramosum* (*C. ramosum*) in gnotobiotic mice increased the gene expression of the passive glucose transporter 2 (*Glut2*) in jejunum and ileum, but not that of *Slc5a1* [55] (Figure 2). However, further studies including the measurement of glucose fluxes are needed to assess the impact of microbes on monosaccharide absorption and the ensuing consequences for the development of metabolic diseases in the host.

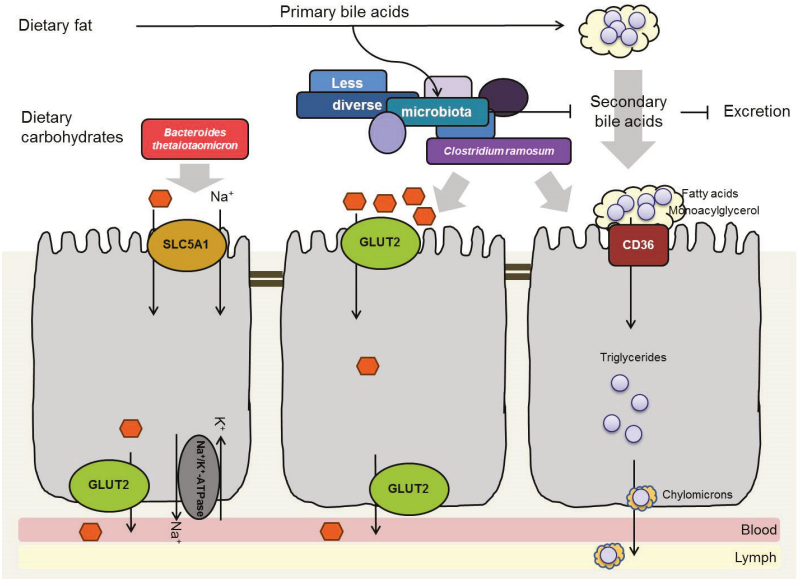

Figure 2. Hypothetical scheme displaying possible contributions of intestinal microbiota to obesity development.

7. Influence of Intestinal Bacteria on Lipid Absorption

Bile acids are synthesized in the liver and secreted into the small intestine where they solubilize dietary lipids through micelle formation. The emulsification increases the surface of the lipids and makes them accessible to lipolytic enzymes that cannot enter lipid droplets. Thereby, bile acids promote the cleavage of lipids resulting in the liberation of monoacylglycerol and fatty acids, which are subsequently absorbed by enterocytes. Bacterial deconjugation and dehydroxylation of bile acids lead to the formation of secondary bile acids, which are less effectively reabsorbed from the ileum and therefore excreted to a greater extent than primary bile acids [56,57]. These modifications catalyzed by the gut microbiota change the physicochemical properties of the bile acids. Possible consequences for the host include a less efficient micelle formation and a diminished lipid digestion and absorption [58]. Therefore, it may be hypothesized that the less diverse intestinal microbiota reported for obese subjects produces less secondary bile acids (deconjugated, dehydroxylated) and consequently, that high concentrations of primary bile acids promote dietary lipid emulsification, digestion and absorption (Figure 2). Indeed, microbial transformation of bile acids is weaker in obese than in lean mice [33]. However, whether bile acid transformation by bacteria in the small intestine causes diminished lipid absorption is doubtful.

Indeed, experiments by Rabot *et al.* (2010) are in conflict with this explanation because they showed that the intestinal microbiota promotes lipid absorption: High-fat diet-fed conventional mice excreted 40% less lipids in their feces than high-fat diet-fed germfree mice [29], the opposite of what would have been expected if conjugated bile acids promoted a more effective lipid absorption. The increased lipid absorption observed in the conventional mice contributed to a higher food efficiency and an increased obesity compared with the germfree mice [29]. In a zebrafish model, it was shown that gut bacteria facilitate the absorption of long-chain and medium-chain fatty acids as well as intracellular lipid droplet formation in enterocytes [59]. Metabolites produced by a Firmicutes strain, which had been isolated from the zebrafish intestine, increased the number of lipid droplets in enterocytes. In contrast, metabolites produced by a Bacteroidetes strain or a Proteobacteria strain did not exhibit this effect [59]. This finding indicates that certain gut bacteria may affect intestinal lipid absorption and lipid droplet formation, whereas others do not. Moreover, the fact that conventional mice display significantly higher small intestinal levels of fatty acid translocase (CD36) than germfree mice suggests that intestinal bacteria mediate an increase in gene and protein expression of this lipid transporter [60]. The relevance of these findings for the development of metabolic disease is not yet clear because research into the role of bacteria in lipid absorption is hampered by the fact that the underlying mechanism is not well understood. In particular the role of proteins such as CD36, fatty acid binding protein (FABP) and fatty acid transport protein 4 (FATP4) in long-chain fatty acid absorption is not entirely clear. The high expression of these proteins in the small intestine and their apical location in enterocytes suggest a role in lipid transport across the brush-border membrane. However, studies using mice deficient in the respective proteins reported conflicting results. The difficulty in gaining a better understanding of their role in lipid absorption and diet-induced obesity development could in part be due to the fact that deletion mutants devoid of any of these genes do not display a specific phenotype because their functions can be compensated by other proteins. Such compensatory adaptations protect genetically modified mice from impaired lipid absorption [61], but impede research on the impact of intestinal bacteria on small intestinal lipid uptake. Currently, administration of fluorescently or radioactively labeled long-chain fatty acids to wild-type gnotobiotic mice seems to be the most promising technique to unravel bacterial effects on intestinal lipid metabolism.

8. Impact of Intestinal Microbiota on Regulation of Host Energy Metabolism

When energy intake exceeds energy consumption fat becomes deposited in adipose tissue. Adipogenesis induced by high-fat diets is enhanced when lipoprotein lipase (LPL) is overexpressed in adipose tissue. Under such conditions, the uptake of fatty acids from plasma into adipocytes, their esterification into triglycerides, and finally triglyceride deposition in adipocytes exceeds lipolysis

of triglycerides as well as their release from adipocytes and their oxidation in various tissues [62]. The gut microbiota has been proposed to affect fat storage by influencing the level of the circulating angiopoietin-like protein 4 (Angptl4) [26], a secreted glycoprotein [63], which is a downstream target of the nuclear peroxisome proliferator-activated receptor family. ANGPTL4 is also known as fasting-induced adipose factor (FIAF) because fasting causes an upregulation of ANGPTL4 in white adipose tissue and liver [64,65]. ANGPTL4 is a lipoprotein lipase (LPL) inhibitor, which regulates the deposition of triglycerides in adipocytes [66]. By way of inhibiting LPL, ANGPTL4 diminishes lipolysis of triglyceride-rich lipoproteins resulting in increased plasma triglyceride levels and subsequently in a decreased uptake of fatty acids into body tissues [67]. Accordingly, mice over-expressing ANGPTL4 display reduced white fat stores [68]. Conversely, suppression of ANGPTL4 stimulates triglyceride storage in adipose tissue [62]. ANGPTL4 mRNA levels are highest in adipose tissue and liver but this mRNA is also found in other tissues, including small intestine and hypothalamus, but at lower levels [64]. Secretion of ANGPTL4 in the intestine was proposed to substantially contribute to its abundance in plasma [26]. This proposition was based on the observation that intestinal ANGPTL4 mRNA levels in conventional mice were twofold lower than those in germfree mice. This led to the conclusion that the gut microbiota represses intestinal ANGPTL4 secretion resulting in decreased levels of circulating ANGPTL4 and consequently in less inhibition of LPL, *i.e.*, increased LPL activity and fat accumulation. In accordance with this interpretation, the relative increase in body fat in conventional *versus* germfree mice was considerably lower in *Angptl4*$^{-/-}$ mice than in wildtype mice. These results were interpreted to mean that germfree mice are generally protected from diet-induced obesity because they display higher ANGPTL4 levels resulting in LPL inhibition and consequently in reduced fat deposition [26]. Another study also observed higher mRNA levels of ANGPTL4 in intestinal mucosa of germfree *versus* conventional mice, but the plasma protein levels of ANGPTL4 between the two mouse groups did not differ [30]. Similarly, administration of *Lactobacillus paracasei* strain F19 to conventional or germfree mice led to increased plasma ANGPTL4 levels and reduced fat accumulation [69]. This suggests that intestinal bacteria do not necessarily lower circulating ANGPTL4 levels as proposed [26], but that at least some members of the gut microbiota exert opposite effects. These observations and other considerations led to the conclusion that the intestinal mucosa does not contribute substantially to circulating ANGPTL4 levels and, therefore, intestinal ANGPTL4 does not play a role as an LPL inhibitor in adipose tissue of germfree mice [70].

Besides providing additional energy, SCFA also fulfill a regulatory function in host energy metabolism. Acetate, propionate, and butyrate are ligands of the G-protein coupled receptors FFAR2 (free fatty acid receptor 2) and FFAR3 (formerly GPR43 and GPR41); these receptors are expressed in ileal and colonic enteroendocrine L cells, adipocytes and immune cells [71]. Upon activation of FFAR3 by SCFA, adipocytes secrete leptin [72] and enteroendocrine cells secrete peptide YY (PYY) [73]. Both hormones reduce appetite [74]. In primary murine colonic cell cultures acetate and propionate enhance the secretion of glucacon like peptide 1 (GLP-1) by enteroendocrine L cells [75]. GLP-1 stimulates insulin production by pancreatic beta cells, improves insulin sensitivity and promotes satiety. Mice lacking *Ffar2* or *Ffar3* display low GLP-1 levels and an impaired glucose tolerance, suggesting that SCFA play an important role in glucose homeostasis [75].

In a recent human intervention study, propionate delivery to the colon was accomplished by oral intake of 10 g of inulin esterified with propionate [76]. Propionate delivered in this way led to increased levels of plasma PYY and GLP-1 accompanied by a reduced energy intake. When the administration of the inulin-propionate ester (10 g/day) was extended to 24 weeks, weight gain, abdominal adipose tissue and hepatic lipid content were decreased and insulin sensitivity was beneficially influenced compared with the inulin control [76]. However, contrary to the observation made after the ingestion of one dose of the inulin-propionate ester, no differences in PYY or GLP-1 could be detected after administration of this compound over an extended period of 24 weeks compared with the inulin-control. The authors hypothesized that the FFAR2/3 receptor response was desensitized over time and that the observed long-term beneficial effects were not mediated by PYY and GLP-1. Moreover, the role of PYY in obesity

per se is contradictory: On the one hand, PYY has anorexigenic effects that counteract obesity; on the other hand, it slows down gut transit time. A prolonged retention time of dietary constituents results in an extended fermentation of bacterial substrates and a more complete absorption of nutrients and SCFA, both of which could promote obesity [28] (discussed in [77]).

Intriguing studies by Gilles Mithieux and colleagues identified a novel mechanism that helps to explain the beneficial effects of SCFA in the prevention of diabetes [78–80]. Both propionate and butyrate were demonstrated to enhance intestinal gluconeogenesis, which mediates these effects by way of signaling through neural circuits that link the enterohepatic portal system with the brain. In the fasted state approximately 20%–25% of the endogenously produced glucose stems from intestinal gluconeogenesis [80]. The neural system surrounding the portal vein senses the glucose produced by intestinal gluconeogenesis and sends a signal to the brain, which modulates the energy and glucose metabolism. Not only dietary proteins promote intestinal gluconeogenesis but also propionate and butyrate do so by two complementary mechanisms [78]. Butyrate promotes intestinal gluconeogenesis in an FFAR2-independent way: oxidation of this SCFA by enterocytes results in higher ATP levels and, in turn, higher cAMP levels [81]. The latter induce the expression of gluconeogenesis genes. Propionate acts both as a substrate of gluconeogenesis and as an agonist of FFAR3, whose activation enhances gluconeogenesis via a neural gut-brain circuit in the afferent periportal nervous system [78]. Therefore the anti-obesogenic and anti-diabetic effects of fermentable fibers are thought to be mediated by intestinal gluconeogenesis via the fermentation products propionate and butyrate.

9. Role of Low-Grade Inflammation in Metabolic Disease

Obesity and type 2 diabetes share a common feature, namely the activation of inflammatory pathways [82]. Gut bacteria have been proposed to be involved in the development of low-grade inflammation in obese and diabetic individuals [83] since they produce pro-inflammatory molecules such as lipopolysaccharides (LPS), flagellins and peptidoglycans. The endotoxin LPS is a cell wall component of Gram-negative bacteria, which becomes liberated into the gut lumen upon bacterial cell lysis [70]. High-energy diets, in particular high-fat diets, as well as the obese and the diabetic phenotype are associated with high plasma LPS concentrations in humans and mice [84–87]. Chronic infusion of LPS enhances obesity and pro-inflammatory signaling and also reduces hepatic insulin sensitivity in wildtype mice, while mice deficient in the glycoprotein cluster of differentiation 14 (CD14) are devoid of most of the symptoms induced by high-fat diet feeding or LPS infusion [85]. CD14 binds and presents LPS to the receptor complex Toll-like receptor 4 (TLR4)/myeloid differentiation factor 2 (MD-2), which triggers an inflammatory response of the host to the bacteria [85,88]. Hence, LPS and its downstream signaling cascade might be a causal link between the gut microbiota and metabolic disease.

But how does LPS enter the host? It has been proposed that LPS is taken up together with dietary lipids within chylomicrons or via paracellular transport through tight junctions [89]. Therefore, dietary fat might promote LPS uptake from the intestine. In support of this hypothesis, high-fat diet feeding in mice was reported to impair the gut barrier resulting in increased plasma endotoxin levels. Impairment of the gut barrier was possibly due to the down-regulation of certain tight junction proteins. In these mice the high-fat diet-induced changes were accompanied by changes in the gut microbiota [90]. Whether the dietary pattern *per se* increases intestinal permeability or whether it alters microbiota composition, which in turn impairs the gut barrier function, remains to be clarified. Presumably, an increase in gut permeability in conjunction with an LPS-enriched gut microbiota, both triggered by high-fat diets, facilitate LPS absorption and contribute to the development of low-grade inflammation.

LPS absorption could also be increased by activation of the endocannabinoid system by LPS itself. In mouse macrophages, LPS promotes the formation of anandamide, a physiological ligand of the endocannabinoid receptor 1 [91]. In mice, activation of this receptor increases gut permeability and plasma LPS levels [92]. In conclusion, a high-fat diet may change the intestinal microbiota in favor of LPS containing bacteria, which in turn may cause activation of the endocannabinoid system, associated

with a weakening of the gut barrier and resulting in increased LPS absorption. This vicious cycle could promote the development of low-grade inflammation under conditions of high-fat feeding.

However, a recent study using three mouse strains differing in their genetic background and their propensity to develop obesity did not reveal any effect on gut barrier integrity in response to four weeks of high-fat feeding (48 kJ% plant fat) in any of the mouse strains. Even prolonged high-fat diet feeding for 12 weeks with a higher fat content (60 kJ%) did not impair small intestinal and colonic permeability in BL/6J mice. These mice displayed an intact intestinal barrier function and inconspicuous LPS levels in portal vein plasma even when fed a high-fat diet based on 78 kJ% lard [93]. Furthermore, owing to the fact that Firmicutes, which do not produce LPS, are enriched in obese subjects, LPS as the microbial mediator of endotoxemia in metabolic disorders is counterintuitive. However, Firmicutes produce fructanases, which degrade fructans to fructose. Following its absorption into epithelial cells fructose becomes phosphorylated by ketohexokinase. Subsequent depletion of intracellular ATP and phosphate levels transiently interrupts protein synthesis and/or increases oxidative stress, which in turn may reduce tight junction protein expression and thereby increases gut permeability and endotoxemia [94]. Whether Firmicutes do indeed contribute to an impairment of the gut barrier by this or another mechanism and thereby facilitate the uptake of LPS derived from the diet or from Gram-negative gut bacteria has not yet been investigated.

10. Obesogenic and Anti-Obesogenic Intestinal Bacteria

Apart from phylum level changes, microbiota modifications at the class, family and genus level are reported for obese subjects. For instance, the bloom of Erysipelotrichi in an obese human individual [95] and in obese mice [30,32,37] suggests a contribution of members of this bacterial class to obesity. The presence of a member of the Erysipelotrichi, *C. ramosum*, was linked to symptoms of the metabolic syndrome in women with type 2 diabetes [96]. Another human study confirmed an association between obesity and an increased intestinal abundance of this species [97], suggesting that *C. ramosum* is critically involved in obesity development. Recently the presence of this bacterium in gnotobiotic mice harboring a simplified gut microbiota of human representative species promoted body weight gain and body fat deposition during high-fat diet intervention [55]. The absence of *C. ramosum* from the microbial community reduced the severity of high-fat diet-induced obesity. The mechanism underlying this obesogenic effect possibly involves the up-regulation of genes playing a role in glucose and lipid absorption as well as in intracellular lipid storage in the small intestine (*Glut2*, *Cd36*, *Plin2*) (Figure 2) [55]. However, whether the up-regulation of these genes does indeed result in increased nutrient absorption and whether this causally contributes to obesity as proposed by Woting *et al.* [55] requires further mechanistic research involving the use of labeled glucose and lipids.

Fei and Zhao (2013) described another obesogenic, LPS-containing bacterium, *Enterobacter cloacae* B29 (*E. cloacae*), which they isolated from an obese Chinese patient. When introduced into germfree mice, these mice developed low-grade inflammation and obesity under high-fat diet feeding; these symptoms were less severe in germfree mice fed the same diet [98]. It appears that more than one strain in the complex and diverse human gut microbiota is capable of promoting obesity in mice. Indeed, eight of twelve human gut bacterial strains, including *Bacteroides* strains and one member of the Proteobacteria (*Escherichia coli*), increased adiposity when introduced as monocultures into germfree mice fed a low-fat, polysaccharide-rich diet. The most pronounced effect on adiposity development was observed after associating germfree mice with either *Parabacteroides distasonis* or *Bacteroides vulgatus*, both isolated from a human volunteer [99]. However, the relevance of single bacterial strains on obesity development needs to be verified in more complex microbial communities or even in conventional mice because the obesogenic properties of a single bacterium may get lost when a conventional background microbiota is present, as observed for *E. cloacae* B29 [98].

An anti-obesogenic effect was reported for the mucin-degrading bacterium *Akkermansia muciniphila* (*A. muciniphila*) [100]. High-fat diet feeding *per se* reduced the cell count of *A. muciniphila*, whereas the treatment with oligofructose or grape polyphenols stimulated the growth of *A. muciniphila* in mice and

reduced adiposity and metabolic endotoxemia [100,101]. Also in humans was a high abundance of this species associated with a healthier metabolic status and improvements in insulin sensitivity and blood cholesterol levels after calorie restriction [102]. In support of these studies, treatment of high-fat diet-fed mice with viable *A. muciniphila* improved gut barrier function and reversed diet-induced obesity, insulin resistance and endotoxemia [100]. Concordantly, the oral gavage of *A. muciniphila* to high-fat diet-fed mice improved glucose tolerance, attenuated visceral white adipose tissue inflammation by increasing the number of regulatory T cells and reducing the levels of pro-inflammatory cytokines, and restored the number and density of mucus-producing goblet cells similar to effects that occurred after the administration of the anti-diabetic drug metformin [103]. These studies identified the potential of *A. muciniphila* to restore glucose tolerance under high-fat diet conditions in conventional mice with a complex microbiota.

Probiotic bacteria, in particular species belonging to the genera *Lactobacillus* and *Bifidobacterium*, or *Escherichia coli* Nissle 1917 are being used to prevent or treat gastrointestinal disorders [104]. Therefore, the administration of probiotics might also offer the chance to prevent or even treat obesity and diabetes. However, the reported effects of certain *Lactobacillus* spp. on body weight vary considerably. A meta-analysis indicated that *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Lactobacillus ingluviei* promote weight gain, while the administration of *Lactobacillus plantarum* (*L. plantarum*) and *Lactobacillus gasseri* (*L. gasseri*) is associated with weight loss in obese humans and animals [105]. Oral application of a diet supplemented with two *Lactobacillus* strains (*Lactobacillus curvatus*, *L. plantarum*) to obese mice reduced obesity and improved inflammatory markers in adipose tissue [106] while the administration of *L. plantarum* alone attenuated body weight gain and dyslipidemia in high-fat diet-fed mice [107]. The consumption of fermented milk containing *L. gasseri* significantly reduced BMI, body fat mass, and waist and hip circumference in healthy subjects with large visceral fat depots [108]. These effects were attenuated after the consumption of the milk product was stopped, suggesting that probiotics must be consumed continuously to maintain their anti-obesogenic effects.

The inverse relationship between the size of the bifidobacterial population and the incidence of metabolic disease suggests that *Bifidobacterium* spp. have an anti-obesogenic or anti-diabetic potential [109–111]. Indeed, supplementation of a high-fat diet with oligofructose restores the number of intestinal bifidobacteria and reduces symptoms of metabolic diseases [112,113]. Therefore, bifidobacteria were hypothesized to mediate the oligofructose-induced improvement of various symptoms of the metabolic syndrome. However, in mice associated with a defined microbial community and fed a high-fat diet, the beneficial effects of oligofructose were independent of the presence or absence of *Bifidobacterium longum* [114]. Oligofructose *per se* reduced obesity and improved glucose tolerance. In contrast, mice mono-associated with *Bifidobacterium animalis* (*B. animalis*) and germfree control mice gained less body weight on a high-fat diet compared with mice mono-associated with *E. cloacae*, indicating that *B. animalis* was less obesogenic than *E. cloacae*. Actually, both *B. animals*-associated mice and germfree mice were not protected from diet-induced obesity as they displayed similar body weights (approximately 33 g and 36 g) after 10 weeks of high-fat diet feeding. In conclusion, *B. animalis* does neither promote nor prevent obesity development [98]. Unlike the *B. animalis* strain used by Fei and Zhao, the daily gavage of *B. animalis* ssp. *lactis* 420 to high-fat diet-fed mice as well as the administration to mice of a high-fat diet pre-mixed with *Bifidobacterium breve* B-3 (*B. breve* B-3) alleviated diet-induced obesity [115,116]. Also in humans, the daily intake of a capsule containing the lyophilized powder of *B. breve* B-3 reduced body fat mass [117]. It may be surmised that the anti-obesogenic effect of *Bifidobacterium* spp. is species- or even strain-specific. It also needs to be clarified whether the animal data are of relevance for the human situation.

Taken together, animal studies suggest that species such as *C. ramosum* and *E. cloacae* are associated with symptoms of metabolic disease, whereas species such as *A. muciniphila* and strains of

Lactobacillus spp. and *Bifidobacterium* spp. are linked to beneficial effects. However, it is still unclear how these bacteria trigger the observed effects and by which molecules they are mediated.

11. Conclusions

The digestive tract represents a complex microbial ecosystem. Associations between certain diseases and patterns of microbiota composition are in part inconsistent among studies, and their meaning is mostly unclear. Moreover, methodological and population-based differences between studies are much larger than the biological differences between obese and lean subjects within a given study, highlighting the need for harmonization and standardization of study designs, sample preparation and analysis. However, even though the reported microbial changes in response to interventions are not uniform, the reduced microbial diversity in metabolically diseased patients seems to be a fairly recurrent finding. Which bacterial molecules are absent or present in a less diverse microbiota and thereby mediate the development of metabolic diseases is unknown. Gnotobiotic animal models offer the opportunity to investigate the interaction of potentially obesogenic and anti-obesogenic bacteria and their metabolites with other gut bacteria and the host. Their known microbial status circumvents the drawbacks of a complex and inter-individually different microbiota in conventional animals and humans. Once the molecular mechanisms underlying the obesogenic and anti-obesogenic effects of gut bacteria have been elucidated, investigations in more complex animal models and finally in human subjects will help to clarify the relevance of these findings.

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Section 5:
Nutrients and Oxidative Stress

nutrients

MDPI

Article

Associations between Vitamin B-12 Status and Oxidative Stress and Inflammation in Diabetic Vegetarians and Omnivores

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Abstract: Diabetes is considered an oxidative stress and a chronic inflammatory disease. The purpose of this study was to investigate the correlations between vitamin B-12 status and oxidative stress and inflammation in diabetic vegetarians and omnivores. We enrolled 154 patients with type 2 diabetes (54 vegetarians and 100 omnivores). Levels of fasting glucose, glycohemoglobin (HbA1c), lipid profiles, oxidative stress, antioxidant enzymes activity, and inflammatory makers were measured. Diabetic vegetarians with higher levels of vitamin B-12 (>250 pmol/L) had significantly lower levels of fasting glucose, HbA1c and higher antioxidant enzyme activity (catalase) than those with lower levels of vitamin B-12 ($\leqslant$250 pmol/L). A significant association was found between vitamin B-12 status and fasting glucose ($r = -0.17$, $p = 0.03$), HbA1c ($r = -0.33$, $p = 0.02$), oxidative stress (oxidized low density lipoprotein-cholesterol, $r = -0.19$, $p = 0.03$), and antioxidant enzyme activity (catalase, $r = 0.28$, $p = 0.01$) in the diabetic vegetarians; vitamin B-12 status was significantly correlated with inflammatory markers (interleukin-6, $r = -0.33$, $p < 0.01$) in diabetic omnivores. As a result, we suggest that it is necessary to monitor the levels of vitamin B-12 in patients with diabetes, particularly those adhering to a vegetarian diet.

Keywords: vitamin B-12; oxidative stress; inflammation; vegetarian; diabetes

1. Introduction

Data from the Third National Health and Nutrition Examination Survey (NHANES III) indicated that an overall estimated US adult population prevalence of low serum vitamin B-12 status was 3.2%, and the prevalence increased to 4.4% for those aged >50 years [1]. It is well known that a vegetarian diet may influence the vitamin B-12 status because vitamin B-12 is present in most animal food sources, and the low status of vitamin B-12 among vegetarians that results in hyperhomocysteinemia, elevated red blood cell distribution width and mean corpuscular volume predisposes one to circulatory problems, and may negate the health benefits of the vegetarian diet [2]. Metformin is considered a cornerstone in the treatment of diabetes; it not only showed a beneficial effect on reducing levels of plasma glucose but also on lowering lipids profiles [3]. However, data from NHANES 1999–2006 found the prevalence of vitamin B-12 deficiency to be 5.8% in US adults with diabetes under metformin therapy [4] and therefore suggest that long-term treatment of diabetic patients with metformin may cause a higher risk of developing vitamin B-12 deficiency [5].

Diabetes is considered an oxidative stress and a chronic inflammatory disease [6]. Recent studies have indicated that vitamin B-12 is an antioxidant, and a lower status of vitamin B-12 might be a potential trigger contributing to increase oxidative stress, particularly in patients with diabetes [7–9].

Vitamin B-12 acts as an antioxidant or anti-inflammation agent, which might modulate oxidative stress responses, including those of inflammatory responses [10–14]. To the best of our knowledge, no clinical study has examined the association between vitamin B-12 status and oxidative stress and inflammation, especially in patients with diabetes who adhere to a vegetarian diet. Therefore, the purpose of this study was to investigate the correlations between vitamin B-12 status and oxidative stress, and inflammation in diabetic vegetarians and omnivores.

2. Materials and Methods

2.1. Study Design

The current study was a cross-sectional study. We enrolled 154 (64 male and 93 female) adult patients (aged 20–84 years) with type 2 diabetes from Lee's Endocrinologic Clinic (Pingtung County, Taiwan). The diagnostic criteria for type 2 diabetes were defined as a glycohemoglobin (HbA1c) $\geqslant$6.5%, a fasting glucose $\geqslant$7.0 mmol/L or a 2-h plasma glucose $\geqslant$11 mmol/L during an oral glucose tolerance test (OGTT), as well as the use of anti-hyperglycemic drugs. We excluded patients with liver or renal disease, pregnant women, and using antioxidants or vitamin B-12 supplements. The inclusion criteria for vegetarian subjects were that the subjects consumed no meat or fish, although they were allowed to consume dairy products or eggs, and had maintained a vegetarian diet for at least one year. Fifty-four diabetic vegetarians and 100 omnivores participated in this study. The study was approved by the Institutional Review Board of Chung Shan Medical University Hospital, Taiwan (CSMUH No.: CS12203). Each subject provided written informed consent to participate in the study.

2.2. Anthropometric and Dietary Measurements

We measured blood pressures, body weights, heights, and waist and hip circumferences of each patient, and then calculated the body mass index (BMI) and ratios of waist to hip circumference. Blood pressure was measured after each patient rested for at least 5 min. Dietary intake was assessed by dietitians and used 24-h diet recall. The data of nutrients were analyzed using the Nutritionist Professional software package (E-Kitchen Business Corp., Taiwan). The ages, gender, smoking, drinking, exercise habits, and medications of all subjects were recorded.

2.3. Blood Collection and Biochemical Measurement

Fasting blood specimens were collected in vacutainer tubes without anticoagulant (Becton Dickinson, Rutherford, NJ, USA). The samples were centrifuged at 3000 rpm for 15 min at 4 °C and the serum was separated. Fasting glucose was measured by Roche Performa glucose meters (Accu-chek, Mannheim, Germany) and glycohemoglobin (HbA1c) was measured by Variant II hemoglobin testing system kits (Bio-Rad Laboratories, Inc., California, CA, USA). Serum total cholesterol (TC), triglyceride (TG), low density lipoprotein-cholesterol (LDL-C), and high density lipoprotein-cholesterol (HDL-C) levels were measured using an automated biochemical analyzer (Hitachi-7180E, Tokyo, Japan). The levels of apolipoprotein A-1 (Apo-A1) and apolipoprotein-B (Apo-B) were measured using polyethylene glycerol (PEG) enhanced immunoturbidimetric assays (Siemens Healthcare Diagnostics Inc., New York, NY, USA).

2.4. Serum Vitamin B-12, Oxidative Stress, and Inflammatory Markers Measurements

Serum levels of vitamin B-12 were measured by electrochemiluminescence immunoassay (ECLIA) using a commercially available kit (Cobas®Roche, Basel, Switzerland). Serum oxidized LDL-C (Ox-LDL-C) was measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Mercodia, Uppsala, Sweden) according to the supplier's instructions. Plasma malondialdehyde (MDA) was determined using the thiobarbituric acid reactive substances method, as described by Botsoglou *et al.* [15]. Red blood cells (RBCs) were diluted with 25x sodium phosphate buffer for superoxide dismutase (SOD) and glutathione peroxidase (GPx) measurements

and 250x sodium phosphate buffer for catalase (CAT) measurement. The methods for measuring CAT, SOD, and GPx in RBCs have been previously described [16–18]; these measurements were performed spectrophotometrically at 240 nm, 325 nm, and 340 nm, respectively. Protein contents of RBCs were determined based on the Biuret reaction of the BCA kit (Thermo, Rockford, IL, USA). Antioxidant enzymes activity levels were expressed as unit/mg of protein. All analyses were performed in duplicate and the variations of repeated determinations were within 5% of the same sample. With regard to the inflammatory markers, serum levels of high sensitivity C-reactive protein (hs-CRP) were quantified by particle-enhanced immunonephelometry with an image analyzer (Dade Behring, Deerfield, IL, USA), and serum levels of high sensitivity interleukin-6 (IL-6) were measured by ELISA using a commercially available kit (eBioscience, San Diego, CA, USA).

2.5. Statistical Analysis

The data were expressed as the means and standard deviations (SD), as well as the medians. A Kolmogorov-Smirnov test was used to examine the normal distribution of variables. Student's *t*-test or the Mann-Whitney rank sum test was used to compare mean values for continuous variables between the diabetic vegetarians and omnivores. For the categorical response variables, differences between the two groups were assessed by the Chi-square test or Fisher's exact test. Pearson product moment correlations or Spearman's rank order correlations were used to examine the correlation between serum vitamin B-12 status (a dummy variable of 1 was set for subjects who had higher levels of vitamin B-12; the variable for lower levels of vitamin B-12 was set at 0) and the levels of blood glucose, oxidative stress, and inflammatory markers. Multiple linear regressions were used to examine the correlations between serum vitamin B-12 status (as an independent variable) and blood glucose, oxidative stress, and inflammatory markers after adjustment for gender and age. The cut-off point of serum vitamin B-12 was set at 250 pmol/L based on the medians of vegetarians and the definition of vitamin B12 deficiency (<150 pmol/L) and borderline deficiency (<200 pmol/L) were according on Pawlak [2]. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using SigmaPlot software (version 12.0, Systat, San Jose, CA, USA).

3. Results

3.1. Characteristics and Dietary Intake of Subjects

The characteristics and dietary intake of the subjects are shown in Table 1. The means for age ($p < 0.01$) were significantly higher in the diabetic vegetarians than in the omnivores. The diabetic vegetarians had a significantly lower frequency under the metformin ($p = 0.02$) or statin ($p < 0.01$) therapy and lower levels of FG ($p = 0.06$), TC ($p = 0.07$), and HDL-C ($p = 0.01$) than diabetic omnivores. However, the levels of TG ($p = 0.03$), hs-CRP ($p = 0.01$), and IL-6 ($p = 0.04$) were significantly higher in diabetic vegetarians than in omnivores. Regarding the dietary intake, the diabetic vegetarians had significantly lower values of energy ($p < 0.01$), protein ($p < 0.01$), fat ($p < 0.01$), cholesterol ($p < 0.01$), and vitamin B-12 ($p < 0.01$) intake than the omnivores, even energy adjusted.

3.2. Levels of Vitamin B-12 in the Diabetic Vegetarians and Omnivores

The levels of vitamin B-12 in the diabetic vegetarians and omnivores are shown in Table 2. The diabetic vegetarians had significantly lower levels of vitamin B-12 than the omnivores ($p < 0.01$) as well as those under metformin ($p < 0.01$) or statin ($p < 0.01$) therapy. There was a significantly higher prevalence of vitamin B-12 deficiency in the diabetic vegetarians than in the omnivores. However, the level of vitamin B-12 was not significantly different between males and females, old and young subjects in both vegetarians and omnivores groups.

Table 1. Basic characteristics and dietary intake of subjects.

	Vegetarians (n = 54)	Omnivores (n = 100)	p Values
females (n, %)	38 (70%)	55 (55%)	0.09
age (years)	65.1 ± 11.3 (63.5) [1]	57.7 ± 10.5 (60.0)	<0.01
duration of diabetes (years)	12.0 ± 8.8 (9.5)	9.4 ± 6.1 (8.0)	0.16
body weight (kg)	62.0 ± 12.6 (59.8)	67.6 ± 14.7 (65.5)	0.05
body mass index (kg/m^2)	24.9 ± 6.2 (25.5)	26.5 ± 5.7 (25.5)	0.31
waist circumference (cm)	88.0 ± 9.9 (89.3)	88.1 ± 11.7 (86.5)	0.63
hip circumference (cm)	95.1 ± 8.0 (94.5)	97.3 ± 10.3 (96.0)	0.32
waist to hip ratio	0.95 ± 0.15 (0.90)	0.92 ± 0.13 (0.90)	0.45
physical activity [2]	34 (63%)	67 (67%)	0.23
Metformin therapy (n, %)	39 (72%)	89 (89%)	0.02
Statin therapy (n, %)	26 (48%)	71 (71%)	<0.01
Metformin + Statin (n, %)	21 (39%)	64 (64%)	<0.01
fasting glucose (mmol/L)	7.3 ± 0.9 (6.7)	7.7 ± 2.1 (7.4)	0.06
HbA1c (%)	7.4 ± 1.2 (7.2)	7.7 ± 1.3 (7.6)	0.30
TC (mmol/L)	4.4 ± 0.8 (4.3)	4.6 ± 0.8 (4.6)	0.07
TG (mmol/L)	1.6 ± 1.3 (1.3)	1.4 ± 1.4 (1.1)	0.03
LDL-C (mmol/L)	2.3 ± 0.6 (2.3)	2.4 ± 0.6 (2.3)	0.26
HDL-C (mmol/L)	1.3 ± 1.2 (1.2)	1.4 ± 0.3 (1.4)	0.01
TC/HDL-C	3.6 ± 1.1 (3.4)	3.4 ± 1.0 (3.1)	0.13
Apo-A1 (g/L)	1.3 ± 0.3 (1.3)	1.2 ± 0.3 (1.2)	0.36
Apo-B (g/L)	0.8 ± 0.2 (0.8)	0.8 ± 0.2 (0.8)	0.77
hs-CRP (mg/L)	2.1 ± 2.6 (1.1)	1.5 ± 1.9 (0.8)	0.01
IL-6 (pg/mL)	2.5 ± 1.9 (1.8)	2.0 ± 1.7 (1.5)	0.04
	Dietary intake		
energy (kcal/day)	1410.0 ± 355.7 (1380.6)	1678.1 ± 478.4 (1621.9)	<0.01
protein (g/day)	45.6 ± 16.7 (43.1)	64.4 ± 23.2 (60.0)	<0.01
protein (g/kcal)	1.98 ± 3.25 (1.57)	1.47 ± 0.17 (1.46)	0.04
% of total calories	12.7% ± 3.1% (12.8%)	15.4% ± 3.5% (14.9%)	<0.01
fat (g/day)	38.0 ± 16.1 (36.5)	58.7 ± 26.3 (55.0)	<0.01
fat (g/kcal)	0.79 ± 1.20 (0.60)	0.89 ± 0.38 (0.86)	<0.01
% of total calories	24.0% ± 8.1% (23.1%)	31.0% ± 10.1% (33.0%)	<0.01
carbohydrate (g/day)	226.1 ± 68.2 (205.7)	225.0 ± 74.0 (217.6)	0.97
carbohydrate (g/kcal)	4.53 ± 6.26 (3.59)	3.43 ± 1.21 (3.37)	0.13
% of total calories	63.3% ± 9.4% (62.1%)	53.9% ± 10.5% (52.9%)	<0.01
cholesterol (mg/day)	50.9 ± 96.0 (1.7)	208.5 ± 158.0 (155.3)	<0.01
cholesterol (mg/kcal)	2.57 ± 13.99 (0.02)	3.19 ± 2.48 (2.42)	<0.01
vitamin B-12 (μg/day)	0.4 ± 0.6 (0.2)	5.5 ± 9.3 (2.9)	<0.01
vitamin B-12 (μg/kcal)	0.02 ± 0.07 (0.003)	0.08 ± 0.14 (0.049)	<0.01

[1] mean ± SD (medians); [2] physical activity: individual exercise at least 3 times every week.

3.3. Levels of Metabolic Biomarkers after Stratifying by Serum Vitamin B-12

The levels of metabolic biomarkers after stratifying by serum vitamin B-12 are shown in Table 3. With regard to the levels of blood glucose, diabetic vegetarians with higher levels of vitamin B-12 had significantly lower levels of fasting glucose and HbA1c than those with lower levels of vitamin B-12 (fasting glucose, $p < 0.05$; HbA1c, $p = 0.02$) and omnivores (fasting glucose, $p = 0.01$; HbA1c, $p = 0.04$). With regard to lipids profiles, diabetic omnivores with higher levels of vitamin B-12 had significantly higher levels of TC (TC, $p = 0.02$) and LDL-C (LDL-C, $p = 0.03$) than those with lower levels of vitamin B-12. Diabetic omnivores with higher levels of vitamin B-12 had significantly higher levels of HDL-C ($p = 0.04$) than vegetarians.

Table 2. Serum vitamin B-12 in diabetic vegetarians and omnivores.

	Vegetarians (n = 54)	Omnivores (n = 100)	p Values [2]
Serum vitamin B-12 (pmol/L)	379.4 ± 333.0 (266.1) [1]	497.9 ± 292.7 (416.9)	<0.01
Males	(n = 16) 342.8 ± 339.3 (265.7)	(n = 45) 456.4 ± 294.9 (356.6)	0.02
Females	(n = 38) 394.8 ± 333.7 (271.3)	(n = 55) 531.9 ± 289.1 (480.1)	<0.01
p values [3]	0.58	0.10	
Age ⩾ 65 years	(n = 26) 333.4 ± 197.9 (283.4)	(n = 20) 469.2 ± 309.1 (362.2)	<0.05
Age < 65 years	(n = 28) 340.6 ± 302.9 (235.1)	(n = 80) 505.1 ± 290.0 (421.4)	<0.01
p values [3]	0.55	0.42	
Vitamin B-12 deficiency [4] (n, %)	10 (18.5%)	5 (5.0%)	0.02
Vitamin B-12 borderline deficiency [4] (n, %)	18 (33.3%)	7 (7.0%)	<0.01
Metformin therapy			
Yes	(n = 39) 319.5 ± 194.1 (266.1)	(n = 89) 493.2 ± 296.1 (410.0)	<0.01
No	(n = 15) 312.9 ± 272.1 (222.0)	(n = 11) 535.9 ± 273.6 (480.1)	0.03
p values [5]	0.44	0.55	
Statin therapy			
Yes	(n = 26) 358.2 ± 289.6 (251.0)	(n = 71) 516.5 ± 303.1 (440.7)	<0.01
No	(n = 28) 321.5 ± 249.6 (275.0)	(n = 29) 452.4 ± 264.9 (349.0)	0.02
p values [5]	0.54	0.15	
Metformin + Statin therapy			
Yes	(n = 21) 331.9 ± 179.5 (252.6)	(n = 64) 506.3 ± 307.8 (437.8)	0.01
No	(n = 33) 307.9 ± 244.7 (243.1)	(n = 36) 483.0 ± 267.3 (367.3)	<0.01
p values [5]	0.26	0.60	

[1] mean ± SD (medians); [2] values were compared between vegetarian and omnivore groups; [3] values were compared between gender or age stratification; [4] Vitamin B-12 deficiency: serum vitamin B-12 < 150 pmol/L; Vitamin B-12 borderline deficiency: serum vitamin B-12 < 200 pmol/L; [5] values were compared between drugs users and non-users.

Table 3. Levels of metabolic biomarkers after stratifying by serum vitamin B-12 [1].

	Vegetarians (n = 54)		Omnivores (n = 100)	
	⩽250 pmol/L (n = 25)	>250 pmol/L (n = 29)	⩽250 pmol/L (n = 15)	>250 pmol/L (n = 85)
Blood glucose				
fasting glucose (mmol/L)	7.3 ± 1.8 (6.8)	6.5 ± 1.5 (6.3) *,†	7.6 ± 1.5 (8.1)	7.1 ± 1.5 (7.1)
HbA1c (%)	7.5 ± 1.1 (7.2)	6.8 ± 0.8 (6.8) *,†	7.3 ± 0.8 (7.5)	7.2 ± 0.8 (7.2)
Lipid profiles				
TC (mmol/L)	4.2 ± 0.8 (4.1)	4.6 ± 0.9 (4.6)	4.2 ± 0.7 (4.2)	4.7 ± 0.8 (4.7) *
TG (mmol/L)	1.4 ± 0.7 (1.3)	1.8 ± 1.7 (1.3)	1.0 ± 0.4 (1.0)	1.5 ± 1.4 (1.1)
LDL-C (mmol/L)	2.1 ± 0.5 (2.2)	2.4 ± 0.6 (2.3)	2.1 ± 0.6 (2.0)	2.4 ± 0.6 (2.4) *
HDL-C (mmol/L)	1.3 ± 0.4 (1.2)	1.3 ± 0.4 (1.2) †	1.5 ± 0.4 (1.4)	1.4 ± 0.3 (1.4)
TC/HDL-C	3.4 ± 0.8 (3.1)	3.8 ± 1.3 (3.6)	2.9 ± 0.7 (2.6)	3.5 ± 1.0 (3.3)
Apo-A1 (g/L)	1.2 ± 0.3 (1.3)	1.3 ± 0.4 (1.3)	1.2 ± 0.3 (1.2)	1.2 ± 0.3 (1.2)
Apo-B (g/L)	0.8 ± 0.2 (0.8)	0.9 ± 0.3 (0.9)	0.7 ± 0.2 (0.7)	0.8 ± 0.3 (0.8)

[1] mean ± SD (medians); * values were compared within groups; † values were compared between vegetarian and omnivore groups at the same stratified level of serum vitamin B-12.

3.4. Levels of Oxidative Stress and Inflammatory Markers after Stratifying by Serum Vitamin B-12

The levels of oxidative stress and inflammatory markers after stratifying by serum vitamin B-12 are shown in Table 4. The diabetic vegetarians with higher levels of vitamin B-12 had significantly higher antioxidant enzyme activity (CAT, $p < 0.05$) than those with lower levels of vitamin B-12. The diabetic omnivores with higher levels of vitamin B-12 had significantly lower oxidative stress (MDA, $p = 0.06$) and inflammation (IL-6, $p = 0.04$) than those with lower levels of vitamin B-12 and the vegetarians (MDA, $p = 0.01$; hs-CRP, $p = 0.03$; IL-6, $p = 0.02$). The diabetic omnivores with higher

levels of vitamin B-12 had significantly higher antioxidant enzyme activity (SOD, $p = 0.04$) than the vegetarians.

Table 4. Levels of oxidative stress and inflammatory markers after stratifying by serum vitamin B-12 [1].

	Vegetarians (n = 54)		Omnivores (n = 100)	
	⩽250 pmol/L (n = 25)	>250 pmol/L (n = 29)	⩽250 pmol/L (n = 15)	>250 pmol/L (n = 85)
Oxidative stress				
MDA (μmol/L)	1.6 ± 0.6 (1.3)	1.6 ± 0.3 (1.6)	1.5 ± 0.3 (1.5)	1.4 ± 0.3 (1.4) *,†
Ox-LDL-C (U/L)	33.7 ± 8.2 (31.1)	31.0 ± 4.7 (31.7)	31.8 ± 10.9 (28.1)	33.57 ± 7.2 (33.1)
Antioxidant enzymes				
CAT (U/mg protein)	19.2 ± 6.8 (19.1)	24.6 ± 10.8 (22.0) *	19.3 ± 5.9 (18.0)	25.5 ± 12.4 (22.4) *
SOD (U/mg protein)	18.4 ± 8.1 (17.0)	14.5 ± 5.7 (14.9)	19.6 ± 7.8 (23.1)	19.6 ± 7.9 (18.6) †
GPx (U/mg protein)	20.0 ± 4.3 (19.5)	20.5 ± 4.9 (20.5)	21.2 ± 4.1 (21.6)	20.1 ± 5.1 (19.8)
Inflammatory markers				
hs-CRP (mg/L)	1.5 ± 1.5 (0.9)	2.7 ± 3.2 (1.5)	1.4 ± 1.6 (0.8)	1.2 ± 1.2 (0.8) †
IL-6 (pg/mL)	2.2 ± 1.7 (1.7)	2.7 ± 2.2 (1.8)	2.4 ± 1.7 (2.0)	1.5 ± 0.8 (1.3) *,†

[1] mean ± SD (medians); * values were compared within groups; † values were compared between vegetarians and omnivores groups at the same stratified level of serum vitamin B-12.

3.5. Correlations between Serum Vitamin B-12 Status and Blood Glucose, Oxidative Stress, and Inflammatory Markers

The correlations between serum vitamin B-12 status and blood glucose, oxidative stress, and inflammatory markers are shown in Table 5. A significant association was found between vitamin B-12 status and fasting glucose ($r = -0.17$, $p = 0.03$), HbA1c ($r = -0.33$, $p = 0.02$), oxidative stress (oxidized low density lipoprotein-cholesterol, $r = -0.19$, $p = 0.03$), and antioxidant enzyme activity (catalase, $r = 0.28$, $p = 0.01$) in the diabetic vegetarians; vitamin B-12 status was significantly correlated with inflammatory markers (interleukin-6, $r = -0.33$, $p < 0.01$) in diabetic omnivores. The similar trends of correlations between vitamin B-12 status and blood glucose, oxidative stress, and inflammatory markers additionally adjustment for gender and age (data not shown).

Table 5. Correlations between serum vitamin B-12 status [1] and blood glucose, oxidative stress, and inflammatory markers.

	Vegetarians (n = 54)	Omnivores (n = 100)	Pooled (n = 154)
	r [2] (p Values)		
Blood glucose			
fasting glucose (mmol/L)	−0.17 (0.03)	−0.12 (0.05)	−0.10 (0.03)
HbA1c (%)	−0.33 (0.02)	−0.06 (0.35)	−0.17 (<0.01)
Oxidative stress			
MDA (μmol/L)	0.07 (0.63)	−0.11 (0.06)	−0.07 (0.17)
Ox-LDL-C (U/L)	−0.19 (0.03)	0.09 (0.42)	0.00 (0.98)
Antioxidant enzymes			
CAT (U/mg protein)	0.28 (0.01)	0.17 (0.03)	0.23 (<0.01)
SOD (U/mg protein)	−0.21 (0.12)	0.00 (0.98)	−0.03 (0.70)
GPx (U/mg protein)	0.06 (0.68)	−0.08 (0.43)	−0.02 (0.78)
Inflammation			
hs-CRP (mg/L)	0.19 (0.17)	−0.05 (0.41)	0.03 (0.69)
IL-6 (pg/mL)	0.13 (0.37)	−0.33 (<0.01)	−0.14 (0.02)

[1] serum vitamin B-12 levels > 250 pmol/L defined as 1; ⩽250 pmol/L = 0; [2] correlation coefficients.

4. Discussion

Mounting evidence has shown that the health benefits of a vegetarian diet in diabetic patients can provide a range of natural products and food forms of benefit for blood glucose and lipid abnormalities in diabetes [19–23]. However, in the present study, we found that diabetic vegetarians had a significantly lower vitamin B-12 status, which might be related to an increase the levels of oxidative stress and inflammation. Vitamin B-12 could potentially be a useful antioxidant, because it can stimulate methionine synthase activity and direct reaction with reactive oxygen and nitrogen species, and through a glutathione sparing effect, can modify signaling molecules to decrease oxidative stress [10–14,24,25]. In addition, vitamin B-12 could also act as an anti-inflammation agent through the mechanisms of down regulation of the transcription factor nuclear factor-kappa B (NF-kB), inhibition of nitric oxide synthase, and promotion of oxidative phosphorylation [25–27]. In the present study, we have examined inflammatory and metabolic profiles in patients with vitamin B12 deficiency and without vitamin B12 deficiency, regardless of their dietary habits (data not shown). Diabetic patients with lower vitamin B-12 status had a significantly higher levels of blood glucose (HbA1c, $p = 0.08$) and inflammation (hs-CRP, $p = 0.03$), and significantly lower antioxidant enzymes activity (CAT, $p = 0.02$ and GPx, $p < 0.01$) than those with higher vitamin B-12 status. It appears that if diabetic vegetarians are at a high risk for vitamin B-12 deficiency may increase risk of having lower antioxidant capacity and higher inflammatory status. Thus, we suggest it is necessary to monitor vitamin B-12 status regularly in diabetic vegetarians.

Metformin, the first line drug for treating diabetes, has been reported to potentially decrease vitamin B-12 status [3–5]. We observed a lower vitamin B-12 status in the diabetic omnivores under metformin therapy, but that was not reached statistically significant (Table 2), therefore, in this study, we consider a vegetarian diet might be a major cause of vitamin B-12 deficiency. The dietary reference intakes (DRIs) of vitamin B-12 in Taiwan are similar to those of the Institute of Medicine (IOM) in the USA and is 2.4 μg per day. Vegetarians, lack the rich vitamin B-12 source of animal food, and although our vegetarian subjects were included lacto- and ovo-vegetarians, the median intake of vitamin B-12 was still lower (0.2 μg/day, Table 1) than the DRIs. The dietary guideline for vegetarians in Taiwan suggest that vegetarians could consume plant food containing substantial amounts of vitamin B-12, such as edible algae (dried green and purple lavers) to meet the recommendation dietary intake of vitamin B-12. However, algal vitamin B-12 appears to be inactive in humans [27]. Although we did not find a significantly lower vitamin B-12 status in diabetic patients under metformin therapy, there is enough scientific evidence to recommend the supplementation of vitamin B-12 in diabetes who are being treated with metformin, to reduce the risk of developing neuropathy and its consequences [28]. As a result, we support that patients with diabetes, particularly those adhering to a vegetarian diet should intake vitamin B-12 supplements or vitamin B-12 fortified food could maintain adequate vitamin B-12 status and prevent vitamin B-12 deficiency.

Vitamin B-12 plays a dominant role in the utilization of carbohydrates, and a lower vitamin B-12 status may cause hyperglycemia. *In vitro* experiments have indicated that the level of glutathione as well as enzyme activity are lower in vitamin B-12 deficient animals; as a result, vitamin B-12 is beneficial for the regulation of glucose [29]. Some observation studies from India have shown that a lower vitamin B-12 status during pregnancy was associated with higher maternal and off-spring insulin resistance [30–34]. Those results are also supported in our diabetic subjects; we found the levels of fasting glucose and HbA1c were significantly lower in the diabetic vegetarians who had a higher vitamin B-12 status (Table 3), and vitamin B-12 status was significantly negatively correlated with blood glucose, particularly in diabetic vegetarians (Table 5). Because a lower vitamin B-12 status may correlate with impaired glucose tolerance, it is important to monitor vitamin B-12 status in patients with diabetes, particularly those adhering to a vegetarian diet. With regard to the association between vitamin B-12 status and lipid profiles, in an observation study of diabetes subjects from Europe and India, the authors found a negative association between vitamin B-12 and lipid profiles [34]. However, in the present study, we found diabetic omnivores with higher vitamin B-12 status had higher levels

of lipid profiles (TC and LDL-C) than those with lower vitamin B-12 status (Table 3); we consider vitamin B-12 status to be positively correlated with lipid profiles, which might be due to animal food containing more fat.

The strength of this study was that it was the first clinical study to investigate the correlation between vitamin B-12 status and oxidative stress, and inflammation in diabetic vegetarians and omnivores. Vitamin B-12 status is significantly correlated with oxidative stress and inflammation in the present study. Because vegetarians are at a higher risk for vitamin B-12 deficiency, that might negate the health benefits of a vegetarian diet for those with diabetes. We suggest that diabetic vegetarians should monitor their vitamin B-12 status regularly and use vitamin B-12 supplements if necessary. Further interventional studies are needed to explore the proper dosage of vitamin B-12 supplements for lower oxidative stress and inflammation in patients with diabetes adhering to a vegetarian diet.

5. Conclusions

Vitamin B-12 status is significantly negatively correlated with the levels of blood glucose, oxidative stress (ox-LDL) and positively correlated with antioxidant enzyme activity in diabetic vegetarians; and significantly negatively correlated with the levels of inflammation in diabetic omnivores. We suggest it is necessary to monitor the levels of vitamin B-12 in patients with diabetes, particularly those adhering to a vegetarian diet.

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Author Contributions: Y.J.L. and P.T.L. conceived and designed the study. M.Y.W. and M.C.L. helped to conduct the study and sample analyses. P.T.L. performed the data analyses and wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

HbA1c	glycohemoglobin
Apo	apolipoprotein
CAT	catalase
FG	fasting glucose
GPx	glutathione peroxidase
HDL-C	high density lipoprotein-cholesterol
hs-CRP	high sensitivity C-reactive protein
IL-6	high sensitivity interleukin-6
LDL-C	low density lipoprotein-cholesterol
MDA	malondialdehyde
SOD	superoxide dismutase
TC	total cholesterol
TG	triglycerol
Ox-LDL-C	oxidized low density lipoprotein-cholesterol

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Naringin Reverses Hepatocyte Apoptosis and Oxidative Stress Associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-Induced Metabolic Complications

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Abstract: Nucleoside Reverse Transcriptase Inhibitors (NRTIs) have not only improved therapeutic outcomes in the treatment of HIV infection but have also led to an increase in associated metabolic complications of NRTIs. Naringin's effects in mitigating NRTI-induced complications were investigated in this study. Wistar rats, randomly allotted into seven groups (n = 7) were orally treated daily for 56 days with 100 mg/kg zidovudine (AZT) (groups I, II III), 50 mg/kg stavudine (d4T) (groups IV, V, VI) and 3 mL/kg of distilled water (group VII). Additionally, rats in groups II and V were similarly treated with 50 mg/kg naringin, while groups III and VI were treated with 45 mg/kg vitamin E. AZT or d4T treatment significantly reduced body weight and plasma high density lipoprotein concentrations but increased liver weights, plasma triglycerides and total cholesterol compared to controls, respectively. Furthermore, AZT or d4T treatment significantly increased oxidative stress, adiposity index and expression of Bax protein, but reduced Bcl-2 protein expression compared to controls, respectively. However, either naringin or vitamin E significantly mitigated AZT- or d4T-induced weight loss, dyslipidemia, oxidative stress and hepatocyte apoptosis compared to AZT- or d4T-only treated rats. Our results suggest that naringin reverses metabolic complications associated with NRTIs by ameliorating oxidative stress and apoptosis. This implies that naringin supplements could mitigate lipodystrophy and dyslipidemia associated with NRTI therapy.

Keywords: naringin; NRTIs; metabolic complications; apoptosis; oxidative stress

1. Introduction

The introduction of highly active antiretroviral therapy (HAART) has reduced the morbidity and mortality associated with human immunodeficiency virus (HIV) infections [1–3]. Drug classified as nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs or NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors and fusion/entry inhibitors are traditionally used in the management of HIV infections [4,5]. The current guidelines on administration of HAART recommend a combination of two NRTIs, one NNRTIs or a protease/integrase inhibitor depending on efficacy and the patient's tolerability [4,5]. NRTIs (abacavir, didanosine, lamivudine, stavudine, zidovudine and emtricitabine) act as false substrates that sabotage viral cDNA chain elongation hence inhibiting viral reverse transcriptase activity and consequently limiting viral replication [4].

Zidovudine (AZT) and stavudine (d4T) have historically been included as components of various combinations of NRTIs which serve as backbone of HAART [6]. While AZT has remained important in the prevention of mother-to-child transmission of HIV, d4T has remained relevant

in the economically less privileged countries because of its relative affordability compared to the preferred alternatives [7–10]. High incidences of metabolic side-effects such as lipodystrophy, metabolic syndrome, peripheral neuropathy, myelosuppression, hepatic steatosis and lactic acidosis have been reported in patients using NRTIs [11–15]. Therefore, while antiretroviral agents have reduced the morbidity and mortality associated with HIV infection, there is persistent increase in the prevalence of these metabolic complications which threaten the success obtained so far with HAART treatment.

NRTIs are associated with hepatotoxicities such as, steatosis, steatohepatitis, disorders of lipid regulation, hepatic enlargement and abnormal liver functions, [16,17]. Furthermore, the World Health Organization (WHO) has advocated the phasing out of d4T from the available list of antiretrovirals due to severe hyperlactatemialactic acidosis and hepatotoxicity, compared to other NRTIs [12,16–18].

Although specific mechanisms through which these complications of NRTIs occur are yet to be clearly defined, it has so far been shown that NRTIs inhibit DNA polymerase gamma thereby leading to a depletion of the mitochondrial DNA and subsequently mitochondrial toxicity [19]. This leads to impaired oxidative phosphorylation (OXPHOS) and subsequent oxidative damage to the cellular machinery coupled with a delay in cell cycle progression which eventually result in apoptotic cell death [12]. These effects have been attributed to the binding of NRTI-triphosphates (the active metabolite of most NRTI following intracellular phosphorylation) to the replicating mitochondrial DNA causing termination of the viral chain elongation [19,20]. Marked increase in reactive oxygen species (ROS), malondialdehyde (MDA, an end-product of lipid peroxidation), and carbonyl proteins (an end-product of protein oxidation), coupled with a decrease in the activities of the enzymatic antioxidant proteins consequent upon a disorder in the oxidative phosphorylation process, have been associated with NRTI administration [16,21].

Currently, there are no standard treatment guidelines for these non-progressive but permanent metabolic complications. Withdrawal from and switching of antiretroviral drug regimens, adjunct pharmacotherapy, and surgical interventions, have previously been tried with limited success [15]. Dietary and nutritional therapies have remained viable options that have not been vigorously pursued. Beneficial effects of some currently available antioxidants have been demonstrated using animal models, but are yet to be validated with large-scale clinical trials [22,23]. There is therefore a need to screen drugs with proven antioxidant effects in the management of the attendant complications of NRTIs.

Plant-derived flavonoids such as naringin (4′,5,7-trihydroxyflavone 7-rhamnoglucoside) which are commonly found in citrus fruits have been recommended as beneficial in reducing the risk of diabetes and cardiovascular diseases in predisposed populations [24]. Its free radical scavenging and antioxidant, anti-apoptotic, antihyperglycemic, antimutagenic, anticancer, anti-inflammatory and cholesterol lowering potentials have been demonstrated [25,26]. Since HIV itself causes symptoms which are similar to those of NRTI-induced metabolic complications [22], it becomes cumbersome to differentiate between the effect of any form of intervention on either the NRTIs administered or on the viral pathogenesis. In this study, we created a model of NRTI-induced metabolic complications in the absence of the HIV infection in order to clearly delineate the observed effects of naringin. The present study was designed to investigate the potential of naringin in reversing metabolic complications of NRTIs and to identify possible mechanisms underlying these observed activities of naringin.

2. Materials and Methods

2.1. Experimental Animals

Eight weeks old, male albino Wistar rats (200–250 g) were purchased from and housed within the premises of the Biomedical Resources Unit (BRU) of the University of KwaZulu-Natal, Durban, South Africa. They were placed in well-ventilated standard plastic cages, exposed to 12:12-h light-dark cycle at an ambient temperature of 23 $\pm$ 2 °C and humidity of 55% $\pm$ 5%. Animals were allowed free

access to tap water and were fed with standard rat chow *ad libitum*. Ethical approval for this study was obtained from the Animal Ethics Committee of the University of KwaZulu-Natal (reference number: 008/14/animal) and the animals were handled humanely in accordance with the guidelines provided by the same body.

2.2. Drugs and Chemicals

Naringin, butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), trichloroacetic acid (TCA), guanidine, ethanol, ethyl acetate, 2, 4-dinitrophenylhydrazine (DNPH), phosphoric acid (H_3PO_4), hydrochloric acid (HCl) were purchased from Sigma-Aldrich® chemicals, St. Louis, MT, USA. d4T and AZT from Aspen Pharmacare®, Durban, South Africa, while vitamin E was purchased from PharmaNatura (Pty) LTD Sandton, South Africa.

2.3. Experimental Design

The rats were divided into seven groups ($n = 7$), (Table 1). All drugs were dissolved in distilled water, which served as the vehicle, prior to administration. Rats in groups I, II and III were treated daily with 100 mg/kg body weight (BW) of AZT by oral gavage [27,28], while groups IV, V and VI were similarly treated with 50 mg/kg BW of d4T [29]. Additionally, rats were treated orally with 50 mg/kg BW of naringin (groups II and V) [30] and 45 mg/kg BW of vitamin E, which was served as the positive control in the study, (groups III and VI) [31], respectively. Rats in group VII served as the vehicle-treated control and were given 3 mL/kg BW of distilled water by oral gavage.

On the 56th day of treatment, rats were sacrificed by halothane overdose, blood was collected by cardiac puncture, centrifuged at 3000 rpm for 10 min and plasma samples stored at −80 °C for further biochemical analysis. Liver as well as visceral and mesenteric fat were promptly surgically removed for further analysis.

Table 1. Animal treatment schedule.

Groups	Treatment (Dose; mg/kg/Day)			
	AZT	**d4T**	**Naringin**	**Vitamin E**
I	100			
II	100		50	
III	100			45
IV		50		
V		50	50	
VI		50		45
VII	Distilled Water (3 mL/kg/body weight/day)			

AZT: (zidovudine); d4T: (stavudine).

2.4. Biochemical Analysis

2.4.1. Fasting Plasma Lipid Profile Estimation

Fasting plasma Total Cholesterol (TC), High Density Lipoprotein Cholesterol (HDL) and Triglycerides (TG) were measured by the Olympus AU 600 auto analyzer (Alternative Biomedical Solutions, Dallas, TX, USA).

2.4.2. Liver Thiobarbituric Acid Reactive Substances (TBARS) Assay

TBARS assay was carried out following the modified method of Halliwell and Chirico [32]. Briefly, 100 mg of liver tissues were homogenized in 500 µL of ice-cold 0.2% H_3PO_4 solution and spun at 1600× *g* for 5 min at 4 °C. Subsequently, 200 µL of the supernatant were added to 500 µL of 2% H_3PO_4, 400 µL of 7% H_3PO_4 and 400 µL of BHT/TBA solutions in a set of clean glass test-tubes, respectively. In another set of eight clean fresh test tubes, 200 µL of serially diluted MDA standard was added to 500 µL of 2% H_3PO_4, 400 µL of 7% H_3PO_4 and 400 µL of BHT/TBA solutions,

respectively. Reactions in both sets of tubes were initiated with 200 μL of 1M HCl. All tubes were incubated in a shaking boiling water bath (100 °C) for 15 min and cooled at room temperature. Thereafter, n-Butanol (1.5 mL) was added to each tube and thoroughly mixed and then 200 μL of the top phase transferred to a 96- well micro-plate in triplicates and read at 532 and 600 nm using Spectrostar® micro-plate reader. The plasma MDA concentrations were calculated using an extinction coefficient of 1.56×10^5 $M^{-1} \cdot cm^{-1}$.

2.4.3. Antioxidant Enzyme Activity

Glutathione peroxidase (GPx) activity in the liver of the rats was determined using a commercially available kit by Cayman chemicals, Ann Arbor, MI, USA. Briefly, 10 mg of liver tissues were homogenized in 90 μL of buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM Dithiothreitol and centrifuged for 15 min at 10,000× *g* at 4 °C. The assay was carried out in a 96-well plate with 20 μL of the supernatant, following the manufacturer's instructions. GPx activity was subsequently measured as the rate of decrease in absorbance of $NADP^+$ at 340 nm on a Spectrostar (Micro-plate reader, Los Angeles, CA, USA).

2.4.4. Liver Carbonyl Protein Determination

This was carried out using a commercial kit (Cayman chemicals, Ann Arbor, MI, USA). Briefly, 100 mg of liver tissues were homogenized in 900 μL of phosphate buffer, pH 6.5, containing EDTA and centrifuged at 10,000× *g* for 15 min at 4 °C. Samples containing 200 μL aliquots each of the supernatant from the liver were placed into two clean glass tubes which served as test and control, respectively. To each tube containing either test or control samples, 800 μL of either 0.2% DNPH or 2.5 M HCl, was added respectively. Samples were then incubated at room temperature in the dark for 1 h with intermittent vortexing and were thereafter treated with either 500 μL of 0.2% DNPH or 500 μL of 2 N HCl, respectively. Protein in both tubes was subsequently precipitated by adding 20% TCA, followed by vigorously mixing the contents of each tube, incubating on ice for 5 min and thereafter spinning the contents of each tube at 10,000× *g* for 10 min at 4 °C. The pellets obtained were further suspended in 10% (*w*/*v*) TCA and incubated on ice for 5 min followed by 10 min centrifugation at 10,000× *g* at 4 °C. The pellets obtained in each case were washed three times in a 1:1 mixture of ethyl acetate and ethanol then resuspended in 6 M guanidine hydrochloride and agitated. The contents (220 μL) of each of test and control tubes were transferred in triplicates into a 96-well microtiter plate and absorbance read at 370 nm using a Spectrostar® micro-plate reader (Los Angeles, CA, USA). An extinction co-efficient value of 0.011 was used in determining the concentration of protein carbonyls in each sample.

2.4.5. Western Blot Detection of Apoptotic Proteins

Protein expression of Bcl-2 associated X protein (Bax) and B-cell lymphoma-2 protein (Bcl-2) were detected using the Western Blot technique. Briefly, 100 mg of liver tissue samples were homogenized in 900 μL of ice-cold radio-immunoprecipitation assay buffer (RIPA buffer) containing 1% protease inhibitor cocktail, 150 mM sodium chloride, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 50 mM Tris (pH 8) and spun at 12,000 rpm at 4 °C. The resulting supernatant was carefully transferred into fresh pre-cooled tubes and kept on ice and protein content determined using Bradford method [33]. Samples were adjusted for equal loading and 35 μg each of the denatured protein samples were loaded per well and resolved by electrophoresis in a 10% SDS-polyacrylamide gel at 150 mV for 1.5 h at room temperature. Separated proteins were transferred on to a nitrocellulose membrane at 100 mV for 1 h at room temperature, membrane blocked in a 5% bovine serum albumin in Tris-buffered saline (TBS-T) solution for 1 h at room temperature and thereafter incubated overnight at 4 °C in a 1 in 200 dilution of anti-Bax and anti-Bcl primary antibodies raised in rabbit, respectively. Membranes were washed five times in TBS-T solution followed by incubation in a 1 in 1000 dilution of the appropriate horseradish peroxidase conjugated secondary

antibodies. Membranes were thereafter washed in TBS-T buffer five times, developed with the Lumiglo reagent (Cell Signaling Technology, Inc., Danvers, MA, USA) and visualized with the ChemiDoc imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Image analysis was carried out using the ImageLab® software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.5. Electron Microscopy

Glutaraldehyde-fixed samples were washed three times in phosphate buffered saline and post-fixed in 1% osmium tetroxide. Samples were then dehydrated sequentially in 30%, 50%, 70% and 100% acetone solution and left overnight in resin-acetone solution (1:1). Subsequently, samples were transferred into 100% resin for two hours at room temperature and allowed to polymerize in fresh 100% resin solution at 60 °C for eight hours. Using the LEICA EM UC6 (Leica Microsystems GmbH, Wetzlar, Germany) ultramicrotome, 80 microns liver sections were cut, stained with uranyl acetate and lead citrate and subsequently viewed under the JEOL 1010 (Tokyo, Japan) transmission electron microscope (TEM). Micrographs were subsequently analyzed using the iTEM version 5.2 software by two independent observers who were blinded from the study.

2.6. Statistical Analysis

All results were expressed as mean ± Standard Error of Mean (S.E.M.). Students' *t*-test was used to determine statistical differences between groups using the Graph Pad Prism® Software version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). p Values less than 0.05 were taken as statistically significant.

3. Results

3.1. Effects of Naringin on Metabolic Complications of NRTIs

AZT or d4T administration resulted in significant ($p < 0.05$) decrease in total body weight, increase in abdominal fat mass and liver index (calculated as the ratio of the wet liver weight to the total body weight) compared to controls (Figures 1 and 2A,B; Table 2). However, concomitant administration of naringin with either AZT or d4T, led to a significant ($p < 0.05$) increase in the total body weight in the AZT-treated rats (Figure 1B) and a non-significant increase in the d4T-treated rats compared to AZT- or d4T-only treated rats, respectively (Figure 1A). Significant ($p < 0.05$) reduction in abdominal fat mass and liver index were also observed with either vitamin E or naringin treatment compared to AZT- or d4T-only treated rats, respectively (Figure 2A,B; Table 2). Additionally, AZT or d4T caused dyslipidemia evidenced by significant ($p < 0.05$) increases in plasma concentrations of TG and TC and significant ($p < 0.05$) decrease in plasma HDL concentration. Co-administration of either naringin or vitamin E with either AZT or d4T, significantly ($p < 0.05$) reversed dyslipidemia (Figure 3A,B) compared to AZT- or d4T- only treated rats, respectively. The magnitude of the effects of naringin on the afore-mentioned indices of NRTI-induced metabolic complications was similar to those of vitamin E. However, naringin produced a significantly ($p < 0.05$) greater increase in total body weight among the AZT-treated rats compared to vitamin E (Figure 1B), while the reverse was observed in the d4T-treated rats (Figure 1A).

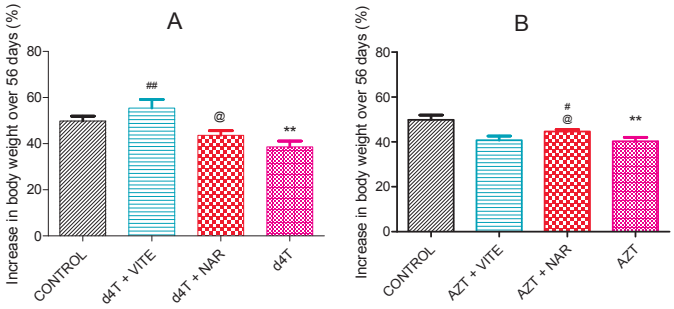

Figure 1. Percentage change in body weight between treatment groups after 56 days of NRTI administration. (**A**) d4T-treated (** $p < 0.01$ compared to control; ## $p < 0.01$ compared to d4T) and (**B**) AZT-treated (** $p < 0.01$ compared to controls; # $p < 0.05$ compared to AZT) rats. @ $p < 0.05$ compared to vitamin E among both d4T and AZT-treated rats.

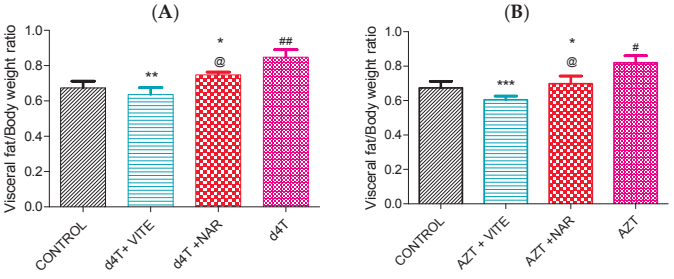

Figure 2. Adiposity index (calculated as a ratio of visceral fat mass to total body weight) among NRTI-treated rats following 56 days of drug treatment. (**A**) d4T-treated (## $p < 0.01$ compared to control; * $p < 0.05$ and ** $p < 0.01$ compared to d4T) and (**B**) AZT-treated (# $p < 0.05$ compared to controls; *** $p < 0.001$ and * $p < 0.05$ compared to AZT) rats. @ $p < 0.05$ compared to vitamin E among both d4T and AZT-treated rats.

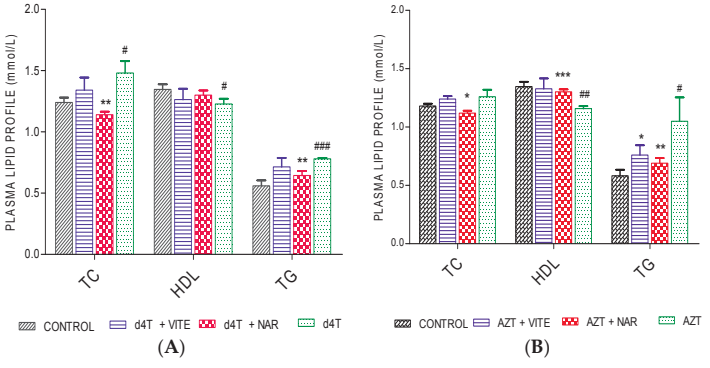

Figure 3. Plasma lipid profile following 56 days of d4T and AZT administration. (**A**) d4T- (** $p < 0.01$ (compared to d4T) and # $p < 0.05$; ### $p < 0.001$ compared to control) and (**B**) AZT- (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to AZT and # $p < 0.05$ and ## $p < 0.01$ compared to control) treated animals after 56 days of drug administrations.

Table 2. Liver and total body weight on day 56; apoptotic index (Bax/Bcl-2 ratio).

Parameters	AZT	AZT + NAR	AZT + VITE	d4T	d4T + NAR	d4T + VITE	Control
Final body weight (g)	307.2 ± 3.7 ^{a}	316.8 ± 1.99 $^{\#}$	303.2 ± 4.2	303.8 ± 4.1 ^{a}	304.2 ± 5.2	315.7 ± 7.5 *	316.6 ± 3.4
Wet liver weight (g)	7.87 ± 0.24 ^{aaa}	6.65 ± 0.17 $^{\#\#\#}$	6.92 ± 0.20 $^{\#\#}$	7.134 ± 0.20 ^{a}	6.45 ± 0.17 *	6.68 ± 0.21 *	6.57 ± 0.20
Liver index (%)	2.30 ± 0.05 ^{a}	2.19 ± 0.02 $^{\#}$	2.44 ± 0.06 $^{\#}$	2.35 ± 0.03 ^{a}	2.11 ± 0.07 **	2.31 ± 0.01 *	2.27 ± 0.03
Bax/Bcl-2 ratio	7.20 ± 0.46 ^{aaa}	1.18 ± 0.11 $^{\#\#\#}$	1.14 ± 0.12 $^{\#\#\#}$	5.42 ± 0.23 ^{aaa}	1.02 ± 0.05 ***	0.76 ± 0.1 ***	3.18 ± 0.27

Values expressed as mean ± SEM. (^{a} $p < 0.05$ and ^{aaa} $p < 0.001$ compared to control; $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ compared to zidovudine; * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ compared to stavudine). NAR (naringin) and VITE (vitamin E). (Liver index was calculated as a ratio of wet liver weight to terminal body weight) × 100.

3.2. Effects of Naringin on NRTI-induced Oxidative Stress

AZT- or d4T-only significantly ($p < 0.05$) decreased glutathione peroxidase enzyme activity and significantly ($p < 0.05$) increased concentrations of MDA as well as carbonyl proteins compared to controls. Co-administration of either naringin or vitamin E, however, significantly ($p < 0.05$) increased glutathione peroxidase activity and reduced the concentrations of MDA and carbonyl proteins (Figures 4, 5 and 6A,B) compared to AZT-only and d4T-only treated rats, respectively. While vitamin E produced more significant ($p < 0.05$) improvements in GPx activity among the AZT-treated rats (Figure 4B), naringin appeared to have produced more significant ($p < 0.05$) decreases in MDA and protein carbonyl concentrations among the d4T- and AZT-treated rats, respectively compared to vitamin E (Figures 5A and 6B).

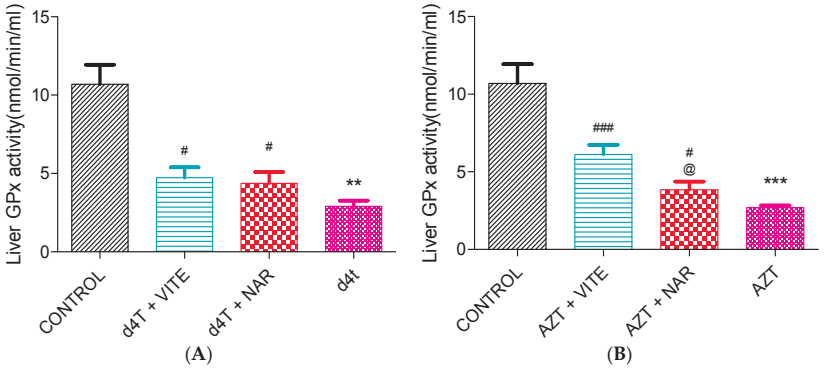

Figure 4. Liver glutathione peroxidase activity following 56 days of drug administration. (**A**) d4T- (** $p < 0.01$ compared to control and # $p < 0.05$ compared to d4T) and (**B**) AZT- (*** $p < 0.001$ compared to control; # $p < 0.05$ and ### $p < 0.001$ compared to AZT) treated animals. @ $p < 0.05$ compared to vitamin E among AZT-treated rats.

3.3. Effects of Naringin on Hepatocyte Apoptosis

Ultrastructural examination of hepatocytes following 56 days of NRTI therapy revealed apoptotic features induced by administration of AZT- or d4T-only. Treatment with AZT-only resulted in condensation, clumping and fragmentation of nuclear chromatin granules with associated damage to the nuclear envelope compared to the control rats (Figure 7A,B), concomitant administration of naringin or vitamin E moderately reduced the nuclear damage compared to AZT-only treated rats as the nuclear envelopes in both groups of rats remained intact (Figure 7C,D). Additionally, treatment with d4T-only resulted in cytoplasmic condensation, severe clumping and fragmentation of the nuclear chromatin materials, increase in the population of apoptotic bodies and phagolysosmes coupled with reduction

in the number of mitochondria in the fields observed (Figure 8A,B). However, co-administration of naringin with d4T minimized nuclear chromatin clumping, nuclear fragmentation and formation of phagolysosmes, maintained mitochondrial population and prevented cytoplasmic condensation (Figure 8C). Similarly, vitamin E prevented cytoplasmic condensation resulting from administration of d4T-only as well as minimized formation of phagolysomes and apoptotic bodies (Figure 8D). These changes were observed to have occurred in a background of intact cellular membrane coupled with preservation of other intracytoplasmic organelles as shown in Figures 7 and 8.

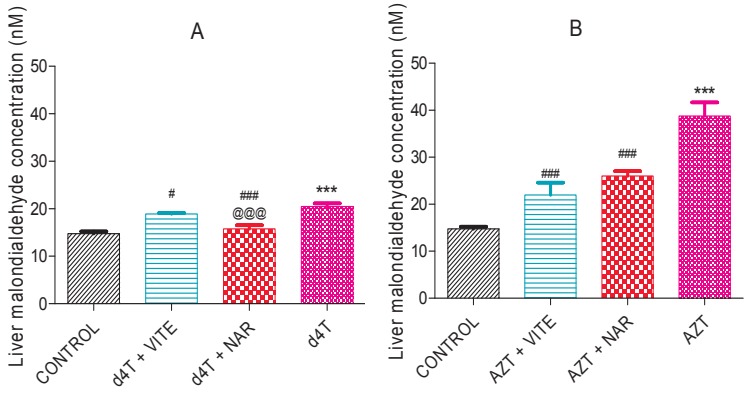

Figure 5. Liver MDA concentrations in: (**A**) d4T- (*** $p < 0.001$ compared to control and # $p < 0.05$; ### $p < 0.001$ compared to d4T and (**B**) AZT- (*** $p < 0.001$ compared to control; ### $p < 0.001$ compared to AZT) treated animals after 56 days of drug administration. @@@ $p < 0.001$ compared to vitamin E among d4T-treated rats.

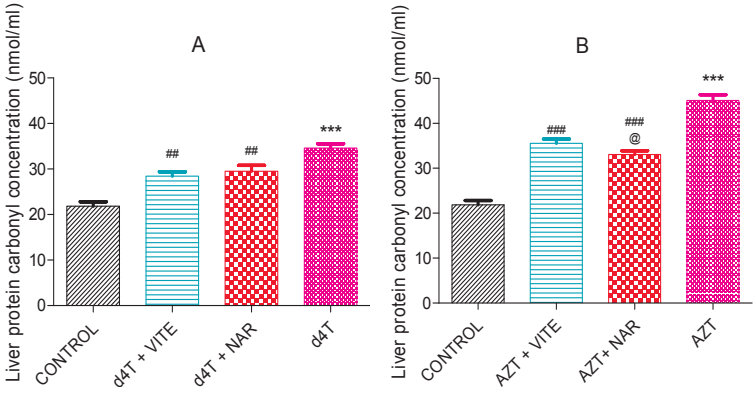

Figure 6. Concentrations of oxidized protein products in the liver after 56 days of drug administration. (**A**) d4T- ((*** $p < 0.001$ compared to control; ## $p < 0.01$ compared to d4T and (**B**) AZT- (*** $p < 0.001$ compared to control; ### $p < 0.001$ compared to AZT) treated animals. @ $p < 0.05$ compared to vitamin E among AZT-treated rats.

Relative quantification of findings from ultrastructural examination was done with reference to the findings on micrographs from the group of control rats and the average of the readings taken by two independent examiners who were blinded from the study were taken into account (Table 3). Treatment with either AZT- or d4T-only, resulted in increased damage to the nuclear

envelope, nuclear fragmentation and nuclear clumping, in addition to increase in the population of phagolysosomes and apoptotic bodies compared to controls. However, co-administration of either naringin or vitamin E caused a relative reduction in damage to the nuclear envelope, nuclear clumping and fragmentation, formation of phagolysosomes and apoptotic bodies which were induced by administration of either NRTI-only.

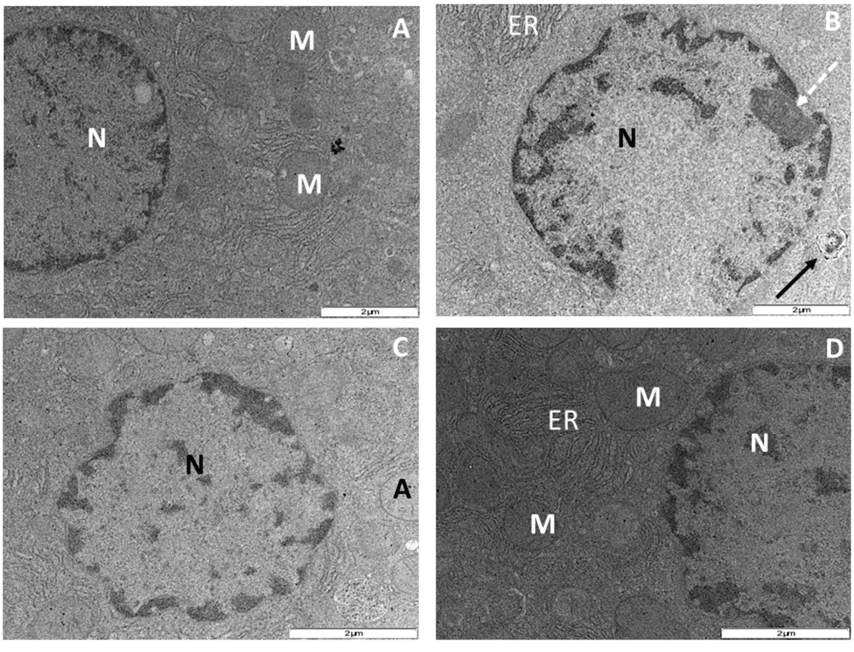

Figure 7. Transmission electron micrograph of rat hepatocytes after 56 days of drug treatment. M = mitochondrion, ER = endoplasmic reticulum and N = nucleus. (**A**) Control rats showing normal nucleus with an even distribution of the nuclear chromatin granules, evenly distributed cytoplasm and preserved mitochondrial architecture; (**B**) AZT-treated rats with markedly reduced mitochondrial population, prominent nucleolus (broken black arrow), clumped and fragmented nuclear chromatin granules coupled with a discontinuation of the nuclear envelope and an apoptotic body (thick back arrow); (**C**) AZT+NAR-treated rats showing minimal clumping of the nuclear chromatin granules, intact nuclear envelope and preserved mitochondrial architecture; (**D**) AZT+VITE-treated rats with intact nuclear envelope, slight clumping of the nuclear chromatin granules in addition to preserved mitochondrial architecture. (Original magnification of reference scale markings: 2 µm = × 15,000).

Table 3. Relative quantification of electron microscopy examination of rat hepatocytes.

Treatment Group	Nuclear Clumping and Fragmentation (% of Control)	Damaged Nuclear Membrane (% of Control)	Phagolysosomes (as a Fraction of the Control)	Apoptotic Bodies (as a Fraction of the Control)
AZT only	+++	+	++	+
AZT + NAR	++	-	+	+
AZT + VITE	++	-	+	+
d4T only	+++	++	+++	++
d4T + NAR	++	-	+	+
d4T + VITE	++	-	+	+
CONTROL	-	-	-	-

+++ ⩾ 50%, ++ = 20% to 50% and + ⩽ 20% relative to control rats. Values recorded are an average of the values obtained by observation of at least five fields from the same sample by two independent examiners who were blinded to the experiment carried out.

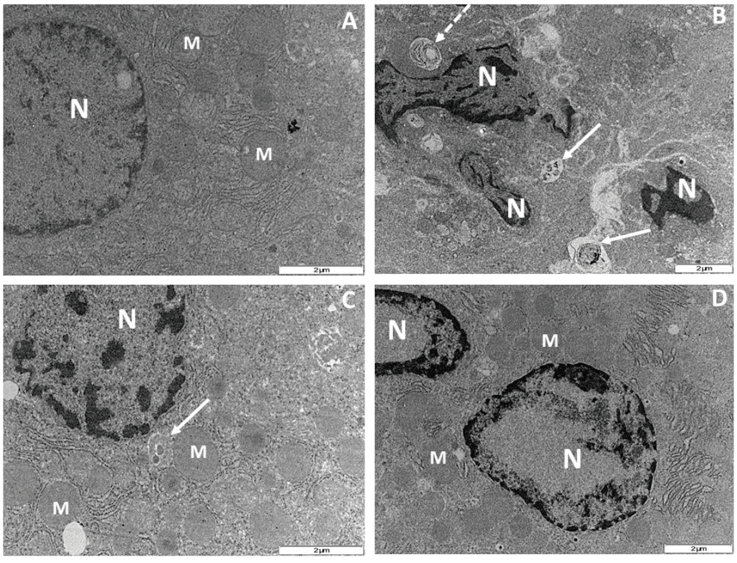

Figure 8. Transmission electron micrograph of rat hepatocytes after 56 days of drug treatment. M = mitochondrion, N = nucleus and ER = endoplasmic reticulum. (**A**) Control rats showing normal nucleus with an even distribution of the nuclear chromatin granules, evenly distributed cytoplasm and preserved mitochondrial architecture; (**B**) d4T-treated rats with severely clumped chromatin granules, broken nuclear membrane and fragmentation of the nucleus. There is an associated condensation of the cytoplasm with appearance of apoptotic bodies (white solid arrows), phagolysosomes (white broken arrows) and sparse mitochondrial population; (**C**) and (**D**) d4T + NAR and d4T + VITE-treated rats, respectively with condensed and fragmented nuclear chromatin granules, numerous mitochondria and even distribution of the cytoplasm. (Original magnification of reference scale markings: 2 μm = × 15,000).

Furthermore, there was significantly ($p < 0.05$) increased expression of Bcl-2 protein and decreased Bax protein expression with co-administration of either naringin or vitamin E with either of the NRTIs (Figure 9). The effect of naringin on Bcl-2 protein expression among d4T- and AZT-treated rats was comparable to the effects of vitamin E (Figure 9C,D). Naringin, however, appeared to have produced a more significant ($p < 0.05$) reduction in the expression of Bax protein compared to vitamin E in AZT- and d4T-treated groups of rats where either naringin or vitamin E was co-administered (Figure 9E,F), respectively. Furthermore, significant ($p < 0.001$) increases in Bax/Bcl-2 ratio arising from AZT or d4T treatment were significantly ($p < 0.001$) reversed by concomitant administration of either naringin or vitamin E (Table 2) with AZT or d4T, respectively.

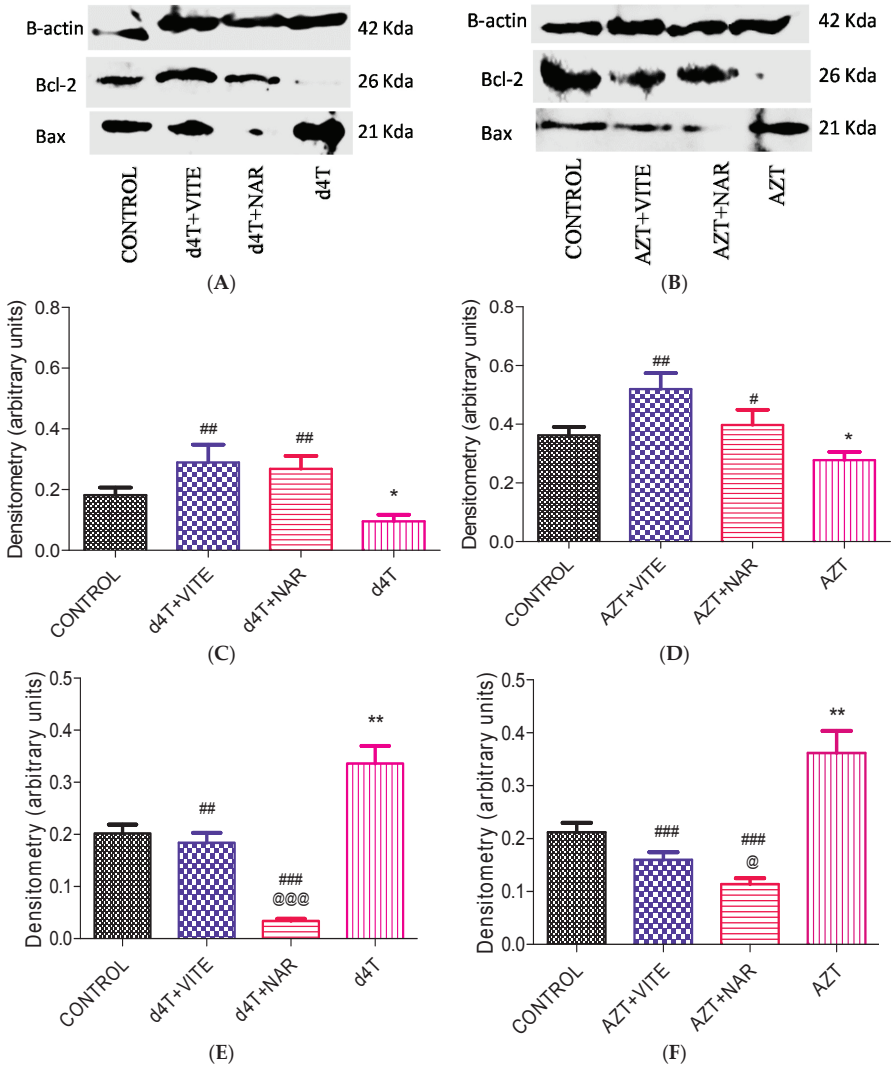

Figure 9. Bax and Bcl-2 protein expression following 56 days of NRTI treatment. (**A**) and (**B**) show Bax, Bcl-2 and beta actin protein expression whilst (**C**), (**D**), (**E**) and (**F**) show the densitometry scans of the respective proteins normalized to the house-keeping protein (beta actin) following 56 days of drug administration; (**C**) and (**E**) d4T- (* $p < 0.05$; ** $p < 0.01$ compared to control and ## $p < 0.01$; ### $p < 0.001$ compared to d4T); (**D**) and (**F**) AZT- (* $p < 0.05$; ** $p < 0.01$ compared to control and # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ compared to AZT). @@@ $p < 0.001$ and @ $p < 0.05$ compared to vitamin E in d4T and AZT-treated rats, respectively.

4. Discussion

Known metabolic complications of NRTI administration include lipodystrophy, dyslipidemia, hepatotoxicity, hepatomegaly, metabolic syndrome, hyperlactatemia, and cardiomyopathy [11,13,34–36]. Cellular oxidative damage caused by mitochondrial toxicity is one of the numerous scientific mechanisms

that underscore the development of these complications [22,37]. Common antioxidants such as vitamins C and E, uridine as well as carnitine have been investigated in preventing or reversing these complications with minimal success [38–40]. Therefore, further screening of newer and perhaps more efficacious antioxidants in managing these complications becomes necessary. Naringin is a readily available and cheap dietary flavonoid present in most citrus fruits with proven antioxidant and anti-apoptotic properties which have been demonstrated in *in vitro*, *in vivo* and *ex vivo* animal models [41–43]. Its candidacy in the management of NRTI-induced metabolic complications is worth investigating.

In this study, we established a model of NRTI-induced metabolic complications, investigated possible mechanisms involved and probed the usefulness of naringin, compared to vitamin E, in ameliorating these complications. Presence of lipodystrophy, evidenced by significant increase in the adiposity index (Figure 2), dyslipidemia (Figure 3) and hepatic enlargement (Table 2), in the presence of significantly reduced total body weight (Table 1) in AZT or d4T-treated rats, were taken as markers of NRTI-induced metabolic complications [44–46]. At the doses administered in this present study, AZT and d4T have previously been shown to exert toxic effects ranging from steatosis, hyperbilirubinemia, hypoproteinemia, ultrastructural damage to the liver as well as the neurons, and oxidative stress [27,29]. Co-administration of naringin with either AZT or d4T, significantly reversed these metabolic complications similarly to vitamin E, as evidenced by significant improvements in the total body weight, reduction of the hypertriglyceridemia and hypercholesterolemia as well as increasing plasma HDL concentrations.

An imbalance between the production of reactive oxygen species (ROS) and intracellular antioxidant capacity underlies the development of oxidative injury which forms the basis for the development of many pathologic conditions [47]. A decrease or an increase in the activities of enzymatic antioxidant proteins (manganese superoxide dismutase (MnSOD) and GPx) has consistently been noted during oxidative stress [48,49]. An unchecked increase in ROS within the cell eventually results in lipid peroxidation, oxidative protein and nucleic acid damage [50,51]. These damages to the cellular framework ultimately cascades into inhibition of cellular enzyme activity and activation of the mechanisms for programmed cell death which eventually leads to cellular demise [50–52]. In this study, NRTI-treated rats exhibited significant increases in MDA (Figure 5) and carbonyl proteins (end products of intracellular oxidative damage to lipids and proteins) (Figure 6) concentrations similar to the findings of Banerjee *et al*. [16]. These were observed against a background of a significant decrease in the activity of glutathione peroxidase. Increased oxidative stress can lead to a reduction in antioxidant enzyme activity due to reduction in the gene expression or suppression of antioxidant proteins' production by oxidative stress [40]. NRTIs have been shown to cause a reduction in antioxidant protein gene expression [21]. A picture of significantly reduced glutathione peroxidase activity (Figure 4) coupled with significantly raised MDA (Figure 5) and carbonyl proteins concentration (Figure 6), suggests a state of overwhelming oxidative stress following NRTI treatment which was significantly improved by concomitant administration of either naringin or vitamin E with AZT or d4T, respectively. Naringin, as an antioxidant, has previously been shown to improve antioxidant gene expression and antioxidant enzyme activity at the dose it was administered in the present study [41,53,54]. On the other hand, vitamin E has previously been administered at various doses for its ability to prevent lipid peroxidation in various tissues [31,55,56].

Pro-apoptotic effects of NRTIs have also been demonstrated previously [35,57]. Increased Bax protein expression, reduced Bcl-2 protein expression, increased Bax/Bcl-2 ratio, and ultrastructural apoptotic changes such as karyorrhexis, karyolysis and formation of apoptotic bodies, in a background of preserved architecture of other intracellular organelles, have been used as markers of apoptosis [58–60]. Furthermore, electron microscopy findings of these ultrastructural changes are regarded as the "gold standard" in identifying apoptosis [61]. In the present study, naringin was observed to have mitigated AZT or d4T-induced apoptosis within the liver tissue comparably to vitamin E as evidenced by a significant reduction in the expression of the pro-apoptotic protein Bax (Figure 9E,F) and a significant increase in the expression of the anti-apoptotic protein Bcl-2

(Figure 9C,D). Bax is a cytosolic protein, which when activated by apoptotic triggers such as ROS [62], translocates to the outer mitochondrial membrane. This causes a reduction in the mitochondrial membrane potential, mitochondrial membrane leakage, activation of caspases and other pro-apoptotic agents, leading to an increase in the rate of apoptosis within the tissue. Bcl-2 on the other hand, prevents apoptosis by binding to and inhibiting pro-apoptotic proteins such as Bcl-2 homology domain 3 protein (BH3) [63]. Antioxidant intake has been associated with an altered rate of cellular death [32] and indeed naringin has previously been shown to possess anti-apoptotic properties [41,64]. Apoptosis plays an important role in the development of some of these metabolic complications of NRTIs and amelioration of the same is required to minimize these complications. From the present study, naringin appeared to have minimized the metabolic complications of NRTIs in a similar fashion to vitamin E's effects in addition to reducing oxidative stress and apoptotic changes. This finding therefore suggests that naringin may be beneficial in such cases of NRTI-induced complications wherein oxidative stress and apoptosis play important roles in their pathogenesis. Furthermore, in d4T-treated rats, we observed the presence of phagocytic lysosomes (a marker of an ongoing autophagic process) [65]. Conversely, naringin or vitamin E co-treatment appear to reduce the development of phagolysosomes and ultrastructural changes indicative of apoptosis, thus further lending credence to the anti-apoptotic effects of naringin. Our study therefore suggests that co-administration of naringin, a dietary flavonoid, together with NRTIs, mitigates AZT or d4T-induced metabolic complications, oxidant stress, apoptosis and autophagy similarly to vitamin E.

Although this study provides preliminary evidence of potential amelioration of metabolic complications of NRTIs, by naringin, it is not clear whether naringin's effects are due to its action on mitochondrial structural defects and function and further investigation of mitochondrial morphology and function is needed. Mitochondrial population reduction, cristae fragmentation, lamellar degeneration, swelling and outer membrane disruptions are some of the reported features of NRTI-induced ultrastructural changes that might contribute to mitochondrial dysfunction and metabolic complications of NRTIs [66,67]. Furthermore, NRTI-induced mitochondrial toxicity is suggested to underlie the development of metabolic complications associated with the use of these drugs [22]. Therefore, a thorough investigation of mitochondrial ATP generation, lactate levels, mitochondrial calcium concentration and specific markers of mitochondrial dysfunction such as uncoupling proteins (UCP-2) and mtDNA depletion and/or mutation would provide a better understanding of naringin's mechanism of ameliorating NRTI-induced metabolic complications. Moreover, the contribution of endoplasmic reticulum (ER) stress to mitochondrial dysfunction has recently become apparent and it is currently believed that the ER and mitochondria are structurally and functionally related [68,69]. Therefore, a dissection of NRTI effects on ER stress in relation to mitochondrial dysfunction might have provided us with a deeper insight into the role of naringin in alleviating metabolic complications of NRTIs. Therefore, future studies would endeavor to look at these parameters in order to provide a fuller understanding of the role of naringin in alleviating NRTI-induced metabolic complications.

5. Conclusions

Naringin's reversal of some of the NRTI-induced metabolic complications provides preliminary evidence of its potential in mitigating NRTI-induced metabolic complications. The mechanism by which naringin ameliorate these metabolic complication possibly involves its antioxidant and/or anti-apoptotic effects. However, a better understanding of its role in the pathophysiology of NRTI-induced metabolic complications and mitochondrial dysfunction needs to be further evaluated.

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Section 6:
Diet, Dyslipidemia and Obesity

nutrients

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Article

Low Urinary Iodine Concentrations Associated with Dyslipidemia in US Adults

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Abstract: Iodine is an essential component of the thyroid hormone which plays crucial roles in healthy thyroid function and lipid metabolism. However, the association between iodine status and dyslipidemia has not been well established at a population level. We aimed to test the hypothesis that the odds of dyslipidemia including elevated total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol and apolipoprotein B, and lowered high-density lipoprotein (HDL) cholesterol and HDL/LDL ratio are associated with urinary iodine concentration (UIC) in a population perspective. Data of 2495 US adults (⩾20 years) in the National Health and Nutrition Examination Survey 2007–2012 were used in this study. Two subgroups (*i.e.*, UIC below *vs.* above the 10th percentile) were compared of dyslipidemia as defined based on NCEP ATP III guidelines. The differences between the groups were tested statistically by chi-square test, simple linear regressions, and multiple logistic regressions. Serum lipid concentrations differed significantly between two iodine status groups when sociodemographic and lifestyle covariates were controlled (all, $p < 0.05$). Those with the lowest decile of UIC were more likely to be at risk for elevated total cholesterol (>200 mg/dL) (adjusted odds ratio (AOR) = 1.51, 95% confidence interval (CI): 1.03–2.23) and elevated LDL cholesterol (>130 mg/dL) (AOR = 1.58, 95% CI: 1.11–2.23) and lowered HDL/LDL ratio (<0.4) (AOR = 1.66, 95% CI: 1.18–2.33), compared to those with UIC above the 10th percentile. In US adults, low UIC was associated with increased odds for dyslipidemia. Findings of the present cross-sectional study with spot urine samples highlight the significant association between UIC and serum lipids at population level, but do not substantiate a causal relationship. Further investigations are warranted to elucidate the causal relationship among iodine intakes, iodine status, and serum lipid profiles.

Keywords: iodine; serum lipids; cholesterol; dyslipidemia; NHANES

1. Introduction

Iodine is an indispensable component of thyroid hormone biosynthesis and normal thyroid function [1]. Thyroid hormone plays a key role in the regulation of multiple mechanisms, particularly lipid synthesis and absorption [2]. Since every cell in the body is affected by thyroid hormone, iodine status is associated with various health outcomes including goiter, hypothyroidism, mental retardation, and dyslipidemia [3]. Impaired lipid metabolism may be resulted from inadequate thyroid hormone production due to insufficient iodine [4]. In an iodine deficient population, serum thyroid stimulating hormone (TSH) levels is elevated to prompt an uptake of circulating iodine by the thyroid [5]. It has been reported that elevated TSH has a positive association with risk for dyslipidemia [6]. Additionally, abnormal thyroid hormone due to iodine deficiency may lead to adverse aftereffects such as hypothyroidism throughout various life stages [7]. It is well-known that hypothyroidism is a major risk factor for the development of dyslipidemia, hypercholesterolemia and cardiovascular disease (CVD) [8,9]. Hypothyroidism accelerates elevations in serum total cholesterol and low-density lipoprotein (LDL) cholesterol by increasing cholesterol absorption in the intestines

and lowering LDL cholesterol clearance from the serum. Moreover, hypothyroidism may increase serum triglycerides by decreasing lipoprotein lipase activity [10,11].

Dyslipidemia broadly describes abnormalities associated with serum lipid metabolism. Its clinical consequences are elevated levels of total cholesterol, triglycerides, and LDL cholesterol, and decreased high-density lipoprotein (HDL) cholesterol levels [12]. Dyslipidemia is one of the risk factors of CVD and a main component of metabolic syndrome. Increased social burdens resulting from CVD and metabolic syndrome have led to extensive investigations to prevent them in the field of nutrition [13,14]. Dyslipidemia consisting of elevated total cholesterol and LDL cholesterol has been observed in patients with hypothyroidism [15,16]. The majority of previous studies noted that abnormalities in total cholesterol and LDL cholesterol were normalized after the successful treatment of hypothyroidism [17,18]. Although many studies examined the association between thyroid dysfunction and lipid levels, we still have no adequate information regarding the relationship between iodine status and serum lipid levels. Only a very few studies have investigated the development of dyslipidemia in relation to iodine status. Uncontrolled and small scale studies have been conducted on the effects of iodine treatments in goitrous subjects with CVD and lipid abnormalities. Previous studies reported that iodine treatments for subjects with goiter resulting from iodine deficiency improved serum lipid profiles by lowering previously elevated total cholesterol and LDL cholesterol [19,20]. In a recent study with mice, hypothyroidic function due to insufficient iodine intake changed lipid profiles, while excessive iodine intake had a beneficial effect on lipid metabolism [21].

Considering that the prevalence of both iodine deficiency and dyslipidemia still remain high among some subgroups in the US [22,23], further research is appropriate to elucidate the association between iodine status and lipids. It takes a long time to develop dyslipidemia from hypothyroidism [11,24] and hypothyroidism from iodine deficiency [25]. If dyslipidemia is associated with inadequate iodine status, the correction for iodine status may have considerable benefits for prevention and treatment of dyslipidemia. Therefore, we hypothesized that urinary iodine concentration (UIC) is associated with the odds of dyslipidemia in the US population. The specific aims of our study were to identify sociodemographic and lifestyle variables affecting UIC and serum lipids, to determine the association of UIC with serum lipid levels, and to estimate risks for dyslipidemia by UIC using recently available National Health and Nutrition Examination Survey (NHANES) data collected from 2007 to 2012. UIC from a spot urine sample is the only indicator of iodine status that is currently available in the NHANES. UIC is a reliable indicator for the iodine status assessment, because more than 90% of dietary iodine appears in urine [26]. UIC from ten repeat collections from spot urine samples or 24-h urine samples can be applied to as a measurement for individual's iodine status, whereas single spot urine samples should be used to assess iodine status only at a population level [22,27]. Although it is hard to conclude anything at the individual level due to limitation of indicators for iodine status in the NHANES, we can describe an association between below *vs.* above the 10th percentile of UIC subgroups with serum lipid concentrations based on large population-based analyses.

2. Methods

2.1. Data Source and Study Sample

The NHANES, a cross-sectional examination survey, is conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC). Each NHANES survey is based on a complex, stratified, multistage, and probability cluster designed to obtain nationally representative samples of civilian, noninstitutionalized residents in the US [28]. The NHANES consists of interviews, laboratory tests, and physical examinations administered by highly trained staff [29]. NHANES protocols were approved by the NCHS Research Ethics Review Board and all subjects aged $\geqslant$18 years participated voluntarily after giving their informed consent [30].

Detailed descriptions of survey plan and design have been previously provided in the NHANES analytic guidelines [28] and thus will not be explained here.

In 1999, the NHANES became a continuous survey with collecting data in every two-year cycles. In this study, we merged three continuous NHANES cycles (2007–2008, 2009–2010, and 2011–2012) and focused on 9164 US adults (⩾20 years) who participated in the NHANES 2007–2012 and have urinary iodine information. We excluded those who were pregnant and lactating women (n = 267), not eligible for data analyses because of missing information on serum lipids (n = 4871), those who were taking any medications for thyroid dysfunction or dyslipidemia (n = 824), and those with missing information on key sociodemographic and lifestyle characteristics (n = 707). The final analytic sample consisted of 2495 adults.

2.2. Definition of Dyslipidemia

We utilized NHANES serum laboratory information on total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, and apolipoprotein B levels to assess dyslipidemia. For this study, dyslipidemia was defined based on the guidelines provided in the third report of the US National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III) [31]: total cholesterol >200 mg/dL for elevated total cholesterol; triglycerides >150 mg/dL for elevated triglycerides; HDL cholesterol <40 mg/dL in men and <50 mg/dL in women for lowered HDL cholesterol; LDL cholesterol >130 mg/dL for elevated LDL cholesterol; HDL/LDL ratio <0.4 for lowered HDL/LDL ratio; apolipoprotein B >130 mg/dL for elevated apolipoprotein B.

2.3. Iodine Status

The NHANES has included measured UIC to monitor the iodine status among US population aged 6 years and older since 1971. Information on UIC has been collected through spot urine samples by the Elemental Analysis Laboratory of the CDC's Division of Laboratory Sciences. UIC was measured by inductively coupled plasma mass spectroscopy (ICP-MS) based on the method by Caldwell *et al.* [32,33] using the same equipment at the same laboratories [34,35]. To examine the association of UIC and serum lipid levels, the participants included in this study were divided into deciles according to UIC distribution and the lowest decile of them was defined as having low UIC (9.0–47.2 μg/L). Then, serum lipid levels were compared between two subgroups (below *vs.* above the 10th percentile of UIC) [36].

2.4. Covariates

The NHANES included information collected on the sociodemographic and lifestyle characteristics through interviews administered by trained interviewers. In this study, variables included in the statistical analytic models were sex (men and women); age (20–39, 40–59, and ⩾60 years); race/ethnicity (non-Hispanic whites, non-Hispanic blacks, all Hispanics, and others); education (less than high school, high school, and more than high school); family poverty income ratio (PIR) (low, ⩽1.85; medium, 1.85 to ⩽3.5; and high >3.5) [28]; supplement use (reported use of a dietary supplement in the last 30 days; yes and no) [37]; smoking (serum cotinine level; low, <0.015 mg/L; medium, 0.015 to <10 mg/L; high, ⩾10 mg/L) [38]; alcohol consumption (average number of drinks/day; no drink, >0 to <1, 1 to <2, and ⩾2 drinks/day) [37]; BMI (underweight, <18.5 kg/m^2; normal, 18.5 to <25 kg/m^2; overweight, 25 to <30 kg/m^2; and obese, ⩾30 kg/m^2) [39]; physical activity (Metabolic Equivalent Task (MET)-minutes/week) from leisure-time physical activity: no activity, 0 to <500, 500 to <1000, and ⩾1000 MET-minutes/week [40].

2.5. Statistical Analyses

We analyzed all data with SAS (version 9.3, SAS Institute Inc, Cary, NC, USA). To account for complex survey design, survey nonresponse, and planned oversampling, we used SURVEY procedure including sample weight, stratum (SDMVSTRA), and primary sampling unit (SDMVPSU) recommended by NCHS for the NHANES analysis [29].

The chi-square test was performed to investigate the distributions (%) and the association between UIC and sociodemographic and lifestyle variables (categorical variables) (Table S1). We used bivariate methods based on simple linear regressions to test the differences in serum lipid profiles by sociodemographic and lifestyle variables (Tables 1 and 2). The variables that were significantly associated were considered as covariates in subsequent analyses. Serum lipid levels including total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, HDL/LDL ratio and apolipoprotein B (continuous variables) were compared between two subgroups (*i.e.*, below *vs.* above the 10th percentile of UIC) using linear regression (Table 3 and Table S2). Multiple logistic regression analyses were used to determine risks for abnormal lipid levels according to UIC (Table 4). Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated after controlling for covariates in two models: unadjusted (model 1) and adjusted for sex, age, race/ethnicity, education, income, supplement use, smoking, alcohol consumption, BMI, and physical activity (model 2). Two-sided p values were considered to be statistically significant if $p < 0.05$.

Table 1. Unadjusted serum lipid status biomarker levels by sociodemographic variable categories for US adults, NHANES 2007–2012 [1].

Sociodemographic Variable		TC	TG	HDL-C	LDL-C	Apo B
		mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
Sex	Men	196.8 ± 1.3 [2,*]	130.5 ± 2.3 **	48.9 ± 0.5 **	121.8 ± 1.2	95.1 ± 0.9 *
	Women	201.6 ± 1.5	112.0 ± 2.6	59.8 ± 0.7	119.4 ± 1.3	91.4 ± 1.0
	r^2, % [3]	<1	2.12	11.31	<1	<1
Age	20–39 years	184.8 ± 1.4 **	112.8 ± 2.8 **	51.6 ± 0.7 **	110.6 ± 1.3 **	85.9 ± 0.9 **
	40–59 years	208.3 ± 1.7	129.0 ± 2.1	54.7 ± 0.8	127.8 ± 1.6	98.7 ± 1.1
	⩾60 years	212.6 ± 2.7	123.8 ± 3.1	59.9 ± 1.2	127.9 ± 2.3	98.3 ± 1.3
	r^2, %	9.80	1.41	3.06	6.20	6.85
Race/ Ethnicity	NHW	200.5 ± 1.3 *	122.0 ± 2.2 **	54.7 ± 0.7 **	121.4 ± 1.1	93.4 ± 0.9 *
	NHB	193.5 ± 2.1	97.1 ± 3.9	58.1 ± 1.1	116.0 ± 1.9	90.1 ± 1.7
	All Hispanics	197.1 ± 1.6	133.6 ± 2.4	49.8 ± 0.6	120.5 ± 1.3	95.6 ± 1.1
	Other	197.5 ± 4.1	128.2 ± 9.8	52.8 ± 1.5	119.1 ± 3.2	92.3 ± 3.0
	r^2, %	<1	2.12	1.75	<1	<1
Education	Less than high school	199.0 ± 1.9	127.9 ± 2.5 **	50.5 ± 0.8 **	122.9 ± 1.5	96.7 ± 1.2 **
	High school	200.7 ± 2.1	129.5 ± 3.4	52.0 ± 1.0	122.8 ± 1.8	96.4 ± 1.4
	More than high school	198.6 ± 1.6	116.5 ± 2.3	56.1 ± 0.5	119.2 ± 1.3	91.2 ± 0.9
	r^2, %	<1	<1	2.16	<1	1.20
PIR	Low	198.1 ± 1.6	124.9 ± 2.2	52.8 ± 0.7 *	120.3 ± 1.4	93.8 ± 0.9
	Medium	198.0 ± 2.3	118.1 ± 3.5	53.7 ± 1.0	120.7 ± 2.0	93.5 ± 1.6
	High	200.6 ± 1.7	120.5 ± 2.6	55.7 ± 0.8	120.9 ± 1.4	92.9 ± 0.9
	r^2, %	<1	<1	<1	<1	<1

[1] Data are from the National Health and Nutrition Examination Surveys. All data except for sample size are weighted accounting for the complex study design according to the directions of the National Center for Health Statistics. Biomarker levels represent mean ± SEM. The total n size was 2495. TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; Apo B, apolipoprotein B; NHW, non-Hispanic white; NHB, non-Hispanic black; PIR, family poverty-income ratio (low: 0–1.85; medium: 1.85 < to 3.5; high: >3.5); [2] p values were based on Wald F test, which tests whether at least 1 of the means across the sociodemographic variable categories is significantly different (* $p < 0.05$, ** $p < 0.01$); [3] values for r^2 are based on model 1, simple linear regression, by categories as shown.

Table 2. Unadjusted serum lipid status biomarker levels by lifestyle variable categories for US adults, NHANES 2007–2012 [1].

Lifestyle Variable		TC	TG	HDL-C	LDL-C	Apo B
		mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
Supplement use [2]	Yes	203.2 ± 1.8 [3],**	117.7 ± 2.7	58.1 ± 0.9 **	121.5 ± 1.6	93.7 ± 1.1
	No	197.0 ± 1.1	123.4 ± 1.7	52.2 ± 0.5	120.1 ± 1.0	93.1 ± 0.8
	r^2, % [4]	<1	<1	3.09	<1	<1
Smoking [5]	Low	203.1 ± 2.3	110.9 ± 3.2 **	59.2 ± 0.8 **	121.7 ± 2.2	92.3 ± 1.4
	Medium	197.8 ± 1.3	122.9 ± 2.6	53.6 ± 0.7	119.6 ± 1.1	92.9 ± 0.9
	High	198.5 ± 2.0	126.8 ± 2.5	51.7 ± 1.0	121.5 ± 1.8	94.7 ± 1.3
	r^2, %	<1	<1	2.84	<1	<1
Alcohol consumption [6]	None	202.9 ± 2.1 *	128.7 ± 3.3 *	51.7 ± 1.0 *	125.5 ± 1.6 **	97.0 ± 1.2 *
	>0 to <1 drink/day	197.2 ± 1.3	119.1 ± 1.7	54.0 ± 0.6	119.4 ± 1.1	92.1 ± 0.8
	1 to <2 drinks/day	202.8 ± 3.0	119.5 ± 5.8	57.1 ± 1.6	121.8 ± 2.2	93.9 ± 1.8
	⩾2 drinks/day	202.8 ± 3.7	128.5 ± 6.4	57.5 ± 1.8	119.6 ± 3.8	94.9 ± 2.6
	r^2, %	<1	<1	1.08	<1	<1
BMI [7]	Underweight	189.4 ± 5.2 **	95.2 ± 7.5 **	62.6 ± 3.1 **	107.8 ± 4.7 **	82.5 ± 2.7 **
	Normal weight	193.9 ± 2.0	99.2 ± 1.7	61.3 ± 1.0	112.8 ± 1.5	85.4 ± 1.0
	Overweight	203.1 ± 1.5	128.0 ± 2.9	52.8 ± 0.8	124.8 ± 1.4	96.3 ± 1.0
	Obese	200.9 ± 1.9	139.4 ± 3.1	47.8 ± 0.6	125.2 ± 1.6	99.1 ± 1.1
	r^2, %	1.15	7.22	11.92	3.09	6.40
Physical activity [8]	No activity	200.5 ± 1.2	127.1 ± 2.0 **	52.7 ± 0.6 **	122.4 ± 1.1	95.4 ± 0.7 **
	0 to <500 MET-min/week.	199.9 ± 3.5	121.7 ± 3.2	54.0 ± 1.0	121.7 ± 2.7	93.3 ± 1.6
	500 to <1000 MET-min/week.	199.2 ± 3.6	117.2 ± 5.3	56.7 ± 1.1	119.0 ± 2.6	92.0 ± 2.0
	⩾1000 MET-min/week.	196.5 ± 1.9	114.4 ± 2.6	55.7 ± 1.1	117.9 ± 2.0	90.6 ± 1.2
	r^2, %	<1	<1	<1	<1	<1

[1] Data are from the National Health and Nutrition Examination Surveys. All data except for sample size are weighted accounting for the complex study design according to the directions of the National Center for Health Statistics. Biomarker levels represent mean ± SEM. The total n size was 2495. TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; Apo B, apolipoprotein B; [2] reported taking supplement containing iodine within the past 30 days; [3] p values were based on Wald F test, which tests whether at least 1 of the means across the lifestyle variable categories is significantly different (* $p < 0.05$, ** $p < 0.01$); [4] values for r^2 are based on model 1, simple linear regression, by categories as shown; [5] smoking status defined by a serum cotinine concentration (low: <0.015 mg/L; medium: 0.015 to <10 mg/L; high: ⩾10 mg/L); [6] calculated as average daily number of drinks/day [(frequency × quantity)/365.25]; 1 drink ≈ 15 g ethanol; [7] underweight: <18.5 kg/m^2; normal weight: 18.5 to >25 kg/m^2; overweight: 25 to <30 kg/m^2; and obese: ⩾30 kg/m^2; [8] calculated as total MET (metabolic equivalent task minutes)-min/week from self-reported leisure-time physical activities.

Table 3. Serum lipid profiles by urinary iodine concentration in US women, NHANES 2007–2012 [1].

Serum Lipids	Women	Low UIC, <10th Percentile (n = 148)	UIC ⩾10th Percentile (n = 1008)	p Value
	Age (years)	mean ± SEM [2]	mean ± SEM	
TC	20–39	188.6 ± 6.1	193.2 ± 4.6	0.2699
	40–59	205.6 ± 6.6	207.7 ± 4.5	0.7754
	⩾60	231.6 ± 7.3	215.4 ± 4.3	0.0052 **
TG	20–39	101.1 ± 13.9	90.7 ± 8.1	0.2493
	40–59	110.3 ± 11.2	118.0 ± 10.2	0.3686
	⩾60	82.39 ± 18.3	99.9 ± 10.3	0.2041
HDL-C	20–39	62.5 ± 2.5	62.5 ± 2.2	0.9948
	40–59	65.1 ± 2.8	67.7 ± 1.9	0.2380
	⩾60	67.3 ± 3.8	67.4 ± 2.8	0.9801
LDL-C	20–39	106.2 ± 5.0	102.0 ± 4.3	0.3677
	40–59	118.9 ± 5.4	117.9 ± 4.0	0.8635
	⩾60	151.8 ± 5.6	132.2 ± 3.2	0.0011 **
Apo B	20–39	81.9 ± 5.1	78.9 ± 4.1	0.3305
	40–59	92.4 ± 4.9	95.1 ± 3.8	0.4881
	⩾60	107.8 ± 5.6	96.2 ± 2.9	0.0103 *

[1] Data are from the National Health and Nutrition Examination Surveys. All data except for sample size are weighted accounting for the complex study design according to the directions of the National Center for Health Statistics. The total n size was 2495. UIC, urinary iodine concentration; TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; Apo B, apolipoprotein B; [2] weighted mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

Table 4. Prevalence of dyslipidemia of adults in relation to urinary iodine concentration in US adults, NHANES 2007–2012 [1].

Dyslipidemia	Model	Low UIC, <10th Percentile (n = 249)	UIC ⩾10th Percentile (n = 2246)	p Value [2]
		AOR (95% CI)	Referent	
Elevated TC (>200 mg/dL)	1	1.35 (0.96–1.89)	1.00	0.0834
	2	1.42 (0.98–2.08)	1.00	0.0666
Elevated TG (>150 mg/dL)	1	0.76 (0.51–1.13)	1.00	0.1652
	2	0.93 (0.63–1.37)	1.00	0.7098
Lowered HDL-C (<40 mg/dL in men, <50 mg/dL in women)	1	0.64 (0.47–0.86)	1.00	0.0040 **
	2	0.92 (0.65–1.30)	1.00	0.6329
Elevated LDL-C (>130 mg/dL)	1	1.41 (1.04–1.92)	1.00	0.0283 *
	2	1.63 (1.16–2.28)	1.00	0.0054 **
Lowered HDL/LDL ratio (<0.4)	1	0.88 (0.65–1.19)	1.00	0.3991
	2	1.71 (1.09–2.67)	1.00	0.0199 *
Elevated Apo B (>130 mg/dL)	1	0.98 (0.50–1.91)	1.00	0.9567
	2	0.98 (0.46–2.08)	1.00	0.9505

[1] Data are from the National Health and Nutrition Examination Surveys. All data except for sample size are weighted accounting for the complex study design according to the directions of the National Center for Health Statistics. Multiple logistic regression analysis was performed to estimate odds ratio for dyslipidemia for subjects from the NHANES 2007–2012 in two models: unadjusted (model 1) and adjusted for sex, age, race/ethnicity, education, income, supplement use, smoking, alcohol consumption, BMI, physical activity (model 2). UIC, urinary iodine concentration; AOR, adjusted odds ratio; 95% CI, 95% confidence interval. TC, total cholesterol; TG, triglyceride; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; Apo B, apolipoprotein B; [2] p value obtained from multiple logistic regression model with diagnosis of dyslipidemia as the outcome variables (* $p < 0.05$, ** $p < 0.01$).

3. Results

The sociodemographic and lifestyle characteristics of the study population categorized by UIC are presented in Table S1. Compared to those with UIC above the 10th percentile, those with UIC below the 10th percentile were more likely to be women, have a poorer level of education, and less likely to take iodine-containing supplements and to be obese.

Unadjusted biomarker concentrations of serum lipid according to sociodemographic and lifestyle characteristics are shown in Tables 1 and 2. Among the sociodemographic variables, sex, age, race/ethnicity, and education (except for total cholesterol) were significantly associated with the majority of serum lipid biomarkers, except for LDL cholesterol which was associated only with age (Table 1). Unlike other sociodemographic variables, PIR was associated only with HDL cholesterol levels. Almost all of the lifestyle variables also had a significant relation with serum lipid status biomarkers (Table 2). Particularly, alcohol consumption and BMI were significantly associated with all five serum lipid biomarkers. The lifestyle variables, supplement use and smoking (except for LDL cholesterol and apolipoprotein B), and physical activity (except for total cholesterol and LDL cholesterol) were also significantly associated with most lipid biomarker concentrations. In our bivariate model, sex, age, and BMI explained the largest portion of the variability in the majority of serum lipid biomarkers; and other sociodemographic and lifestyle variables accounted for little variance in most serum lipid biomarkers.

Serum lipid levels by UIC are shown in Table 3 and Table S2. In men, serum lipid levels did not differ according to UIC, whereas in women, two serum lipid biomarkers differed significantly by UIC. Particularly in older women ($\geqslant$60 years), serum lipid levels such as total cholesterol, LDL cholesterol, and apolipoprotein B were significantly different according to UIC (all, $p < 0.05$). Older women with low UIC had a higher level of total cholesterol, LDL cholesterol and apolipoprotein B levels (231.6 $\pm$ 7.3 mg/dL, 151.8 $\pm$ 5.6 mg/dL and 107.8 $\pm$ 5.6 mg/dL, respectively) than those with UIC above the 10th percentile (215.4 $\pm$ 4.3 mg/dL, 132.2 $\pm$ 3.2 mg/dL and 96.2 $\pm$ 2.9 mg/dL, respectively).

The adjusted ORs and 95% CIs for risk factors of dyslipidemia after adjustment for covariates by UIC are described in Table 4. In comparison to adults with UIC above the 10th percentile, adults with the lowest decile of UIC were more likely to have dyslipidemia. The adjusted ORs of dyslipidemia risk factors among adults with low UIC *vs.* UIC at or above the 10th percentile were 1.63 (95% CI, 1.16–2.28) for elevated LDL cholesterol (>130 mg/dL) and 1.71 (95% CI, 1.09–2.67) for lowered HDL/LDL ratio (<0.4). Adults with low UIC had increased risk of elevated total cholesterol (>200 mg/dL) at marginally significant levels ($p = 0.0666$).

4. Discussion

In this study, significant differences were found in multiple lipid measures according to UIC. Majority of adults with UIC above the 10th percentile had desirable serum lipid range, whereas, those with UIC below the 10th percentile had elevated total cholesterol, LDL cholesterol, and apolipoprotein B. Our findings are consistent with previous researches regarding lipid profiles related to iodine status and iodine treatments in European [19,41] and African countries [42]. The research on 136 adolescents with endemic goitrous in Germany demonstrated that those with iodine deficiency-induced goiter had abnormally higher average total cholesterol and LDL cholesterol levels compared to non-goitrous control subjects [19]. In another study conducted by the same research group, the effects of iodine treatment for six months improved serum lipid levels [41]. Iodine supplement treatment for euthyroid goiter on children resulted significant decrease in total cholesterol and LDL cholesterol to their normal level [43]. A recent randomized controlled intervention study also reported that 6-month intervention with iodine supplementation significantly decreased risks for hypercholesterolemia in overweight and obese Moroccan women [44]. Additionally, a rural African population-based study reported that pregnant women and their offspring in an iodine deficient area are more likely to have a risk of coronary complications and hyperlipidemia compared to non-iodine deficient region [42]. Empirical evidence from clinical studies has demonstrated that iodine treatment was successful for those with CVD and

high blood pressure [45]. There have also been many studies on thyroid dysfunction as a risk factor of lipid abnormalities and CVD. In adolescents with type 1 diabetes, subclinical hypothyroidism was positively correlated with mild dyslipidemia and increased the risk of CVD [46]. In 40 Spanish children aged 2–9 years, mean HDL cholesterol level was significantly lower in children with hypothyroidism compared to healthy children [47]. As thyroid failure has become one of determinants for dyslipidemia, an assertion has been raised that biochemical screening for thyroid disorders is needed for every dyslipidemia patient [48]. These existing studies suggest that long-term inadequate iodine intake may increase risks for dyslipidemia and CVD even though no clinical sign of thyroid dysfunction are present. We significantly expanded the scope of prior study using data from the representative US population and found that this association is still significant among US adults.

No association was found between TSH and UIC except for the case of excessive iodine intake where TSH levels were elevated by inhibiting thyroid hormone synthesis, which is known as the Wolff-Chaikoff effect [32,49]. It is hard to find UIC associated with TSH, particularly in the case of low UIC. Low UIC stimulates thyroid auto-regulation and an offsetting increase in serum triiodothyronine (T3), which normalize thyroid function [50]. Therefore, thyroid function test measuring TSH and thyroxine (T4) levels cannot be a good indicator for reflecting iodine status. Even though insufficient iodine intakes assessed by a single spot urine sample is not necessarily consistent with chronic iodine deficiency or subclinical hypothyroidism, persistent iodine deficiency leads to decreased thyroid hormone synthesis and elevated TSH easily with low-normal serum T4 concentration and high-normal serum T3 range [6]. As mentioned earlier, thyroid hormone is closely link to many metabolic pathways. Thus, the decrease in thyroid hormone synthesis induced by inadequate iodine intake raises the possibility of abnormal lipid metabolism [4]. In addition to classic pathway of lipid metabolism related to thyroid hormone, thyroid hormone-independent effect of TSH on lipid abnormalities was observed [51,52]. Lowered activity of hepatic lipase, which plays an important role in modulation of lipid levels and promoting cholesterol uptake by the liver, due to elevated TSH levels directly affect serum lipid levels [53]. In accordance with this mechanism, biochemical responses iodine deficiency may produce a more atherogenic lipid and higher risk for CVD [54]. Although iodine is also an essential component of the thyroid hormone and healthy thyroid function, iodine itself may play an important role in the cardiovascular health such as cancer-fighting properties and anti-inflammation [55]. However, only limited data support the proposition that iodine status was inversely associated with risk for CVD and iodine intakes were the largest contributor to mortalities due to CVD [20]. The underlying mechanism influencing serum lipid abnormalities by iodine status still remains partially unclear. A perspective and population-based study is required to elucidate a mechanism connected to iodine deficiency, thyroid function, and serum lipid. Therefore, continual monitoring of the independent role of iodine status in dyslipidemia allows us to put a new perspective on the treatment and possible prevention of CVD as well as dyslipidemia.

This study has several limitations. An important limitation of the study is that our findings are based on the cross-sectionally designed survey data. Such a fact makes it difficult to determine a causal relationship between UIC and dyslipidemia. However, this study highlighted that those with low UIC had increased risk of lipid abnormalities. In an animal study conducted by Zhao *et al.* [21], they confirmed the decreasing effect of high iodine intake on hyperlipidemia in mice, but did not investigate that kind of effect in humans. Therefore, adding to our results, it will be necessary to make further efforts to identify a casual effect of high iodine exposure on lipid concentration among those with iodine excess. Investigating potential mechanism between a wide range of iodine intake and lipid levels can provide us with a better understanding of the role of iodine status in pathogenesis of lipid metabolism. Another limitation is the lack of key information on iodine intake. Spot urine sample UIC is the only indicator for iodine status measurement in the NHANES. It has been well-documented that UIC obtained from spot urine sample is a suitable indicator for assessment of population's iodine status, but not for individual's [27]. Moreover, assessing iodine status based on UIC could not determine the actual amount of iodine intake from dietary sources. To overcome recognized

limitations, we used the decile approach. Using this approach, we could have significant findings regarding the association between UIC and serum lipids. However, findings from decile grouping of study samples' UIC distribution still have the limitation that inconsistent results can be found with the different populations. Therefore, their association should be interpreted with circumspection. Understanding the impact of actual dietary iodine intakes and individual's iodine status on serum lipids is worth further investigation as well. Lastly, the study using mice reported that the effect of iodine intake on lipid metabolism may vary depending on the sex of the mice, mentioning needs of considering interaction between iodine status and the sex hormone in the lipid metabolism [21]. Further investigation is needed to explore the effect of these interactions. Adding the strengths of the present study mentioned above, this is a recent and unique study investigating the association between UIC and serum lipid profiles based on larger samples of the US adult population. We confirmed that sociodemographic and lifestyle factors affect UIC and serum lipids, the significant association between low UIC and lipid profiles persisted even after controlling for those confounding factors in our analysis models.

5. Conclusions

In summary, according to the NHANES 2007–2012, we found a cross-sectional association between low UIC measured by a spot urine sample and serum lipid profiles, particularly among total cholesterol, LDL cholesterol, and apolipoprotein B. Thus, efforts for maintaining adequate level of urinary iodine are recommended to prevent risk factors for dyslipidemia among US adults. However, there is a limitation in that, even though UIC used in this study as a biomarker might capture iodine status at the population level, but not at the individual level. Therefore, there is a need for further study investigating the relationship between biomarkers for iodine status that can reflect the iodine status of individuals and serum lipid levels. Additionally, further prospective research regarding the effect of iodine status on lipid metabolism and vice versa will establish epidemiological evidence for a causal relationship between them and help develop effective educational and clinical interventions in this area.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/3/171/s1, Table S1: Sociodemographic and lifestyle characteristics of study subjects, NHANES 2007–2012, overall and by urinary iodine concentration, Table S2, Serum lipid profiles by urinary iodine concentration in US men, NHANES 2007–2012.

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Review

Epigallocatechin Gallate: A Review of Its Beneficial Properties to Prevent Metabolic Syndrome

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Abstract: Obesity and being overweight are linked with a cluster of metabolic and vascular disorders that have been termed the metabolic syndrome. This syndrome promotes the incidence of cardiovascular diseases that are an important public health problem because they represent a major cause of death worldwide. Whereas there is not a universally-accepted set of diagnostic criteria, most expert groups agree that this syndrome is defined by an endothelial dysfunction, an impaired insulin sensitivity and hyperglycemia, dyslipidemia, abdominal obesity and hypertension. Epidemiological studies suggest that the beneficial cardiovascular health effects of diets rich in green tea are, in part, mediated by their flavonoid content, with particular benefits provided by members of this family such as epigallocatechin gallate (EGCG). Although their bioavailability is discussed, various studies suggest that EGCG modulates cellular and molecular mechanisms of various symptoms leading to metabolic syndrome. Therefore, according to *in vitro* and *in vivo* model data, this review attempts to increase our understanding about the beneficial properties of EGCG to prevent metabolic syndrome.

Keywords: metabolic syndrome; green tea; epigallocatechin gallate; EGCG; endothelial dysfunction; cardiovascular diseases

1. Introduction

Metabolic syndrome (MS) is a major and growing public-health and clinical challenge worldwide whose affects approximately 25% of the adult in the world [1,2]. MS increases the risks of developing type 2 diabetes (5-fold), stroke (2- to 4-fold), myocardial infarction (3- to 4-fold) and the risk of death (2-fold) regardless of a previous history of cardiovascular events [2,3]. MS is defined by a multitude of pathophysiological disorders comprising abdominal obesity, insulin resistance, high blood pressure, and dyslipidemia. Several scientific organizations have attempted to formulate working definition of the syndrome [2]. Although each definition possesses common features, the major problem with these definitions is their applicability to the different ethnic groups, especially to define obesity cut-offs. This is particularly evident for the risk of type 2 diabetes which is apparent at much lower levels of obesity in Asians compared to Europeans [2]. In this context, the International Diabetes Federation (IDF) proposed a new set of criteria with ethnic specific cut-offs. However, for many years, the most commonly accepted definition is that of the National Cholesterol Education Program Adult Treatment Panel (NCEP ATP III). Thereby, the diagnosis of MS is established when the patient describes at least three of the following criteria: abdominal obesity, hyperglycemia, elevated blood pressure and dyslipidemia. Abdominal obesity is defined by a waist circumference cut-off greater than 102 cm for men and 88 cm for women, and hyperglycemia is defined by a fasting plasma glucose greater than

5.6 mmol/L (100 mg/dL) and/or the existence of a symptomatic treatment (such as metformin or insulin in the most advanced forms). Furthermore, hypertension is diagnosed when patients present a systolic and/or diastolic blood pressure greater than 130 mmHg and 85 mmHg, respectively, and/or specific treatment (angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers). Finally, dyslipidemia is established when plasma triglycerides (TG) are greater than 1.7 mmol/L (150 mg/dL), and/or high-density lipoprotein cholesterol (HDL-C) are lower than 1.0 mmol/L (40 mg/dL) for men and 1.3 mmol/L (50 mg/dL) for women, and/or when patient is already receiving symptomatic treatment (fenofibrate).

Dietary, pharmacological and surgical strategies have been developed in the last decade to prevent metabolic syndrome. Recently, beneficial effects of a polyphenol-enriched diet have been reported in the prevention of this metabolic disease [4]. Polyphenols represent an important group of phytochemicals found in plants and more than 8000 polyphenolic compounds are currently known [5]. According to the number of phenolic rings, polyphenols are classified into four categories: phenolic acids, flavonoids, stilbenes and lignans. Flavonoids represent 60% of dietary polyphenols and they are classified into seven groups: flavones, flavonols, flavanones, isoflavones, flavanols, anthocyanins and chalcones.

Evidence from epidemiologic studies supports a potential role for some flavonoids in the reduction of cardiovascular risk. For instance, flavonoids are able to prevent against endothelial dysfunction through averting oxidation of low-density lipoproteins (LDL) [6], platelet aggregation and adhesion [7], and smooth muscle cell migration and proliferation [8]. Moreover, according to recent data aiming to evaluate association between dietary flavonoid intake and cardiovascular risk through analyses of prospective cohort studies, it has been reported that intakes of epigallocathechin gallate (EGCG) (relative risk: 0.87; 95% confidence interval: 0.80, 0.95) were inversely associated with the risk of cardiovascular diseases [9]. Based on these considerations, this review attempts to (i) describe green tea polyphenols, their main pharmacokinetic properties and theirs structure/activity relationship explaining its antioxidant effects and (ii) to explain the beneficial properties of EGCG to prevent pathological disorders defining MS such as obesity, insulin resistance, dyslipidemia and hypertension.

2. Green Tea Polyphenols

Green tea, derived from the tea plant *Camellia sinensis* is considered as the most consumed beverage in the world [10]. Originally found in China, the tea plant is now cultivated in over 30 countries and it is estimated that about 120 mL per person of tea beverage is consumed every day [11]. According to data obtained by high performance liquid chromatography (HPLC), green tea leaves are composed of 26% fibres, 15% proteins, 2%–7% lipids, 5% vitamins and minerals, secondary metabolites as 1%–2% pigments, 30%–40% polyphenols of which at least 80% flavonoids and 3%–4% methylxanthines [10,12,13]. This composition can vary depending on growing conditions like, geographical location (climate, soil, *etc.*), agricultural practices (fertilizers, deadheading, *etc.*) and the properties of the plant itself (variety, age of the leaf, position of the leaf on the harvested shoot, *etc.*) [10,13].

Tea infusion is as a hot aqueous extraction containing more hydrosoluble compounds than liposoluble derivatives. An increase of time and temperature would theoretically enrich beverage in green tea leaf components. However, it has been reported that the optimum extraction occurs for water at 80 °C and for 5 min to 15 min for green tea leaves in powder or in bag form, respectively. Indeed, degradation of bioactive compounds is suggested beyond these times and temperatures [14].

During the past decade, the health-promoting effects of green tea and its polyphenols have been intensively investigated. Flavonoids are the most important polyphenols in tea leaves. They represent the major component of green tea infusions, with a percentage between 37% and 56% of weight of solid extracts [10]. Furthermore, green tea beverages also contain carbohydrates, amino acids, organic acids, methylxanthines, minerals, polymers and tannins and traces of volatiles compounds (Table 1) (for review see [13]). Catechins are the main flavonoids found in green tea beverage [15]. They are

constituted by a 2-phenylchromane skeleton substituted in 3, 5, 7, 3′ and 4′ positions with hydroxyl groups. During the biosynthesis, if the B-ring derives from the gallic acid synthon, the catechin is also substituted in 5′ position with a hydroxyl group and thus named "*gallo*" catechin. Moreover, the hydroxyl group in 3′ position can be esterified with the gallic acid, thus forming catechin "*gallate*". Finally, the levorotatory (2R, 3R) compounds are considered as "*epi*" catechins while the dextrorotatory (2S, 3R) compounds are simply named "*catechins*". Thus, with these combinations, eight molecular structures can be distinguished (Figure 1).

Among catechins, only EGCG has an interest in the field of medicinal chemistry. Indeed, EGCG is the most abundant catechin in green tea infusions (for review see [13,15]) and it is considered as one of the most active molecules known for their antioxidant properties [16] (Table 2).

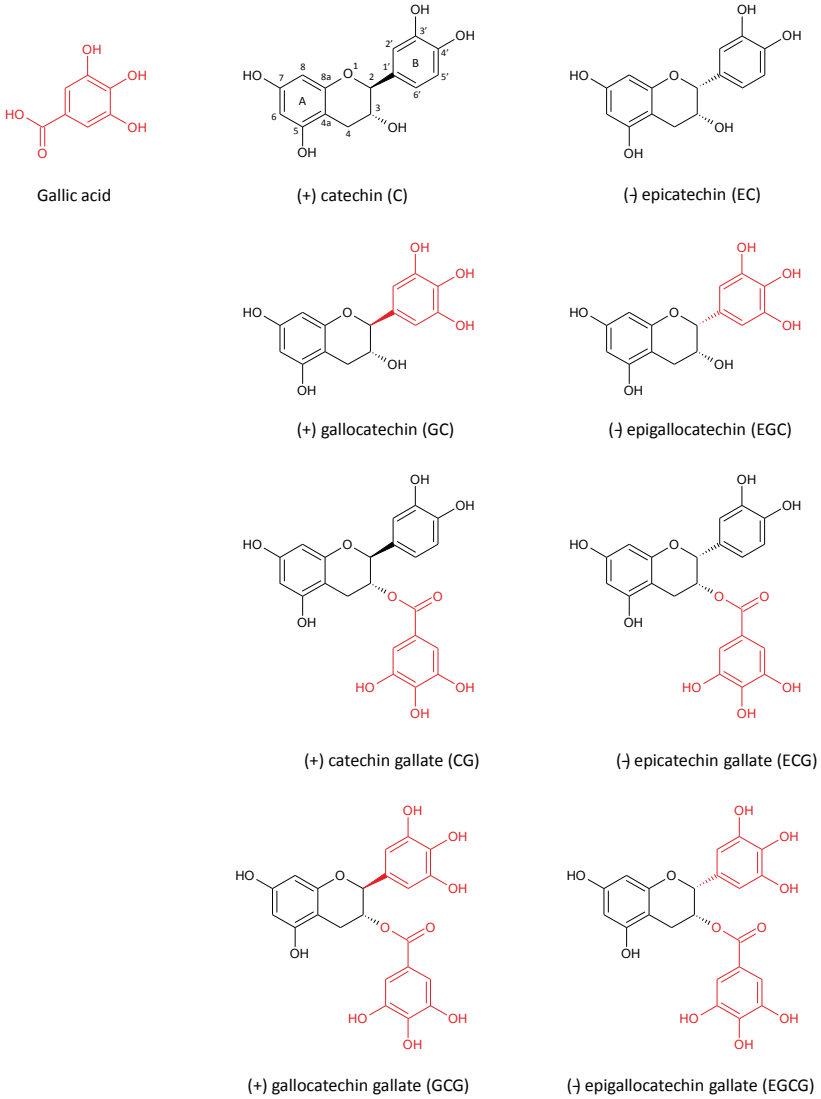

Figure 1. Molecular structure of gallic acid and catechins.

Table 1. Mean composition (% weight of solid extract) of green tea infusion determined by high performance liquid chromatography (HPLC).

Compound	% Weight of Solid Extracts
Flavonoids	37–56
Carbohydrates	10–15
Amino acids	8–12
Organic acids	7.5–9.5
Methylxanthines	7–9
Minerals	6–8
Polymers and tannins	3–4
Volatiles	Traces

Table 2. The composition of polyphenols in green tea leaves determined by high performance liquid chromatography (HPLC) (adapted from [14]).

Catchins	ConntraIion (mg/mL, Mean ± SD)
(+) catechin (C)	19.70 ± 0.10
(−) epicatechin (EC)	123.43 ± 0.13
(+) gallocatechin (GC)	51.10 ± 1.13
(−) epigallocatechin (EGC)	279.87 ± 1.87
(+) catechin gallate (CG)	nd
(−) epicatechin gallate (ECG)	108.55 ± 0.11
(+) gallocatechin gallate (GCG)	3.90 ± 0.06
(−) epigallocatechin gallate (EGCG)	324.54 ± 0.17
TOTAL	911.09

3. Properties of EGCG in the Control of Oxidative Stress

In several *in vitro* studies, EGCG has been found to have the highest antioxidant activity compared to others catechins [17]. Indeed, EGCG has shown an efficient ability in scavenging free radicals species, notably through achievement of the $ATBS^{\bullet+}$ radical scavenging test [17]. One hypothesis to explain these properties is a low reduction potential of EGCG due to its high capacity for giving an electron [16]. Electron delocalization in the molecular structure is described as a property of polyphenolic compounds which could in part be responsible for their antioxidant activity [18]. In the catechin skeleton, the saturation of the heterocyclic ring prevents electron delocalization between the A and the B ring. Thus, for green tea catechins, the antioxidant potential mainly comes from the strong presence of hydroxyl groups in their molecular structures. EGCG, with 8 hydroxyl groups notably in 3′, 4′ and 5′ positions and with a gallate moiety in C-3 is a better electron donor than the others catechins and thus the best scavenger of free radicals species [16,17].

Moreover, the antioxidant activity of EGCG is also due to its ability to chelate metal ions. Troubles in metals homeostasis can lead to an oxidative stress which appears in chronic diseases like diabetes, cardiovascular disease and atherosclerosis [19]. It has been reported that EGCG can chelate metals like iron (Fe) [20], copper (Cu) [21,22], chromium (Cr) [23] and cadmium (Cd) [24,25]. The phenolic groups notably at the B ring are mainly suspected to be responsible for this property [18]. The chelation of metal ions by EGCG is however considered as a minor mechanism in the antioxidant action compared to its free radical scavenging capacity [25,26]. Interestingly, it has been noted that EGCG, in addition to chelate ions, also reduces Fe (III) and Cu (II) in Fe (II) and Cu (I), respectively [27,28]. Fe (II) and Cu (I) are involved in Fenton reaction, with production of radical oxygen species (ROS) [19]. Furthermore, as it is commonly found with antioxidant polyphenols, EGCG may generate ROS *in vitro*, probably via auto-oxidation and dimerization [29–31]. Indeed, Hou Z. *et al.* have proposed a mechanism of EGCG auto-oxidation through a classical pathway including transfer of electron [30]. Therefore, it has been proposed that EGCG is oxidized in EGCG radical (EGCG·) through a sharing of an electron with the oxygen O_2 thus producing superoxide anion (O_2^{-}). Then, this EGCG radical can form a

homo-dimer with another EGCG radical or a dimer radical (dimer·) with another EGCG. Finally, the neutralization of the dimer radical can occur via production of superoxide anion from the O_2. Thus, the conversion of O_2^- in H_2O_2 by the superoxide dismutase (SOD) makes this enzyme indispensable for the inhibition of the propagation of the chain reactions [30] (Figure 2). The 3′,4′,5′-trihydroxy function and the aromatic B ring mainly supports this ability for EGCG to share an electron. Furthermore, while the interaction between SOD activity and EGCG is not clearly established, it has been reported on rats with acetic acid-induced colitis an increases activity of SOD in EGCG-treated rats in comparison with placebo or control rats. To explain these data, authors suggested that the enhanced antioxidant activity of EGCG might be related to its special molecular structure appeared to be important for these actions, which includes two catechol groups, three gallate groups, and two hydroxyl groups [32]. This structure would explain the increase in gene expression induced by EGCG since several studies have reported an increase of *sod* gene expressions induced by this catechin [33,34].

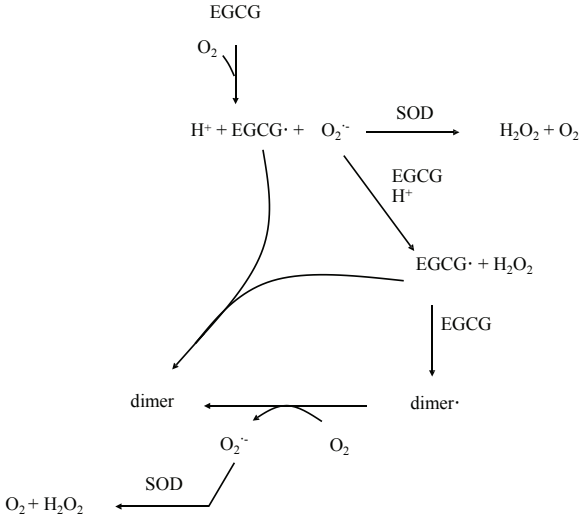

Figure 2. Mechanism of epigallocathechin gallate (EGCG) auto-oxidation and dimerization adapted from [30].

As previously described, EGCG is a natural antioxidant and most of its pharmacological properties are considered to be due to their antioxidant effects. These properties are beneficial to prevent various diseases associated with an increased oxidative stress. However, it has been reported that EGCG has pro-oxidant properties, mainly in cancer cells where it contributes to induce apoptosis [35]. One of the first studies showing the pro-oxidant properties of EGCG has found that 4 mM EGCG is able to favor hydroxyl radical and superoxide anion productions to promote tumor cells apoptosis. Further, it has been reported in this study that copper mediated oxidation of EGCG possibly leads to the formation of polymerized polyphenols. It was indicated that copper oxidized catechins were more efficient prooxidants as compared with their unoxidized forms [28]. Furthermore, toxic effects of EGCG observed *in vivo* following consumption of dietary supplements in humans [36] and administration of tea extracts in animal studies were considered as based on its pro-oxidant activities of EGCG [37]. A study conducted on NCr *nu/nu* mice, xenografted with human lung cancer cells, has demonstrated that intraperitoneal treatment with 30 mg/kg EGCG increases significantly ROS production [38].

4. Pharmacokinetical Properties of EGCG in Humans

Pharmacokinetic parameters of green tea polyphenols, particularly EGCG, have been well investigated in rodents but some of these remain unclear in humans [39]. Few pharmacokinetic studies have evaluated the bioavailability of EGCG. However, it has been revealed a very low absorption of EGCG (probably <5%) and an average T_{max} of 2 h after *per os* administration [40–42]. Green tea catechins are predominantly absorbed intestinally, in the jejunum and the ileum, via a paracellular diffusion through epithelial cells [43]. Once absorbed, EGCG is found in plasma in large proportion (>75%) in a free form [39,44]. The only calculated apparent distribution volume 0.15 L/kg in rat theoretically reveals a weak distribution of EGCG [45]. Despite this low distribution, EGCG seems to diffuse well through tissues in the body. Indeed, EGCG has been found in fetuses and placenta of pregnant rats [46] and in the brain through crossing the blood-brain-barrier [47,48]. EGCG is metabolized on one hand through methylation by the catechol-*O*-methyltransferase (COMT) producing predominantly the primary metabolite di-methoxyl-EGCG (di-OMe-EGCG) [49]. On the other hand, EGCG can be glucurono- and/or sulfo-conjugated [40]. In addition, it is now well established that EGCG can also be metabolized by the intestinal microbiota [50,51]. The half-life is around 3 h, according to the association with others catechins, in a purified form or from tea infusion [40,42]. EGCG metabolites are both excreted through biliary and urinary pathways. However, only traces of EGCG are detected in urine after oral administration [39,45,49,52]. Furthermore, EGCG can be reabsorbed from the intestine through enterohepatic re-circulation.

Although the metabolic transformation of catechins in humans is well understood, relatively little is known about the biological effects of catechin metabolites. However, several studies seem to agree on possible antioxidant properties of both EGCG and its metabolites. Thus, it has been found that *O*-methylated derivatives of (−)-epicatechin are able to inhibit the peroxynitrite-mediated nitrotyrosine formation [53]. Furthermore, in human skin fibroblasts, it has been shown that 3′-*O*-methyl-epicatechin prevents UVA-induced oxidative damage through an enhancement of HO-1 activity [54]. Interestingly, it has been found that HUVEC have the capacity to convert (−)-epicatechin into methyl derivatives, which inhibited NADPH oxidase activity [55].

It has been emphasized that the low bioavailability of EGCG should be considered for the extrapolation of *in vitro* studies to *in vivo* situations. This point is currently debated, notably because *in vitro* studies are often performed with non-physiological concentrations of EGCG [56]. However, numerous factors have been identified to enhance or to diminish its bioavailability [57]. However, the benefits of green tea consumption in humans result from long-term exposition whereas *in vitro* studies supply short-term effects [58]. Thus, *in vitro* data could not necessarily be related with important relevance to clinical data, so it can suggest that *in vitro* studies often converge with epidemiological studies [56,59].

5. Roles of EGCG in Obesity

Obesity is principally the consequence of a positive energy balance driven by increased calorie-dense food consumption and reduced physical activity. Adipose tissue is composed of adipocytes, pre-adipocytes, immune cells and endothelial cells. It can respond rapidly and dynamically to alterations in nutrient excess through adipocytes hypertrophy and hyperplasia [60]. Adipose tissue has long been considered as an organ of lipids storage and mobilization. It has recently been identified as an endocrine organ because of its ability to secrete a large amount of biologically active metabolites as glycerol, free fatty acids (FFA), and pro-inflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6) or leptin [61–63].

FFA are involved in the increase in glucose, triglycerides and VLDL synthesis in adipocytes. In addition, reducing insulin sensitivity of skeletal muscle, FFA inhibit glucose uptake and consequently raise circulating glucose levels which increase the pancreatic insulin secretion and lead to hyperinsulinemia [64]. Secretion of TNFα and IL-6 by adipocyte and macrophage are increased and promotes insulin resistance and lipolysis in adipose tissue [65]. Leptin is a regulator of food intake,

body weight and fat mass. The plasma levels of leptin are positively correlated with the degree of adiposity in healthy and obese individuals [66–68]. Besides, leptin is known to be a NO-dependent vasodilator and an endothelium-independent vasoactive agent [69,70]. Thereby, acute hyperleptinemia induces vasorelaxation that seems to contradict the hypertension observed during obesity. This could be explained by a study showing that leptin receptors in coronary arterioles are downregulated in high-fat fed sedentary mice, leading to endothelial dysfunction [71]. In addition, leptin may impair endothelial function through oxidative stress by increasing the formation of ROS that reduce the bioavailability of NO and upregulate proinflammatory cascades including adhesion and chemotactic pathways in endothelial cells [72–74]. Various studies have described the beneficial properties of EGCG to prevent obesity. Thus, Snoussi *et al.* reported that oral administration of EGCG decoction daily to male Zucker rats fed a high fat diet (22% fat, 43% carbohydrates and 21% proteins) resulted in reduction of body weight within 1 week. In addition, rats treated with EGCG had significantly lowered blood lipids (50% triglycerides and 25% cholesterol) and blood glucose (15%) concentrations. Furthermore, it has been shown that EGCG is able to control glucose homeostasis through a reduction of intestinal SGLT-1/GLUT2 ratio and an enhancement of adipose GLUT4 [75].

Fiorini *et al.* have studied the effects of EGCG on obesity and hepatic steatosis in leptin-deficient *ob/ob* mice. Treatment with 85 mg/kg EGCG for 5 days resulted in decreased body weight gain compared to control mice. EGCG treatment also reduced significantly total hepatic fat content (22.7% $\pm$ 11.0%), increased hepatic energy stores and hepatic antioxidant activity through an enhancement of glutathione level in EGCG-treated mice, compared to control mice. Lipophilic oil red O stain showed that EGCG treatment decreased hepatic steatosis through a significant decrease of palmitic and linoleic acids [76].

Various mechanisms have been proposed to explain the anti-obesity properties of EGCG. Several groups have reported a modulation of dietary lipid absorption by EGCG treatment. In a study conducted on male C57BL/6 mice fed with high fat diet supplemented with EGCG, the anti-obesity properties of this flavonoid was explained by a decreased of food digestibility affecting substrate metabolism of intestinal mucosal and liver, leading to increased post-prandial fat oxidation and reduced incorporation of dietary lipids into tissues [77]. Furthermore, EGCG has been reported to inhibit pancreatic lipase. Thus, in obese C57BL/6 mice fed with high fat diet, it has been found that treatment with 0.32% EGCG for 6 weeks favored a significant decrease in body weight (44%) in comparison with control mice. To explain these beneficial effects, it has been suggested that EGCG is able to inhibit pancreatic lipase [78]. Recently, the molecular interactions between EGCG analogs and pancreatic lipase have been described by Wang *et al.* These authors confirmed that EGCG had different effect on activity, conformation, thermodynamics and kinetics of pancreatic lipase suggesting that EGCG could contribute to the development of natural effective pancreatic lipase inhibitors to prevent human obesity [79]. Otherwise, it has been demonstrated that a high consumption of EGCG inhibited pancreatic lipase *in vitro* and suppressed postprandial serum triglycerides in a dose-dependent manner [80]. To explain the mechanism of action, it has been proposed that the hydroxyl moieties of EGCG interact with the hydrophilic head group of phosphatidylcholine at the exterior of a lipid emulsion by forming hydrogen bonds. These interactions may lead to formation of cross-links followed by covalescence of the emulsion droplets [81]. Several studies have examined the effects of EGCG on fat metabolism and particularly in β-oxidation. Recently, it has been suggested that EGCG could alleviate fat deposition in broilers through inhibiting fat anabolism and stimulating lipid catabolism in broilers. Then, the supplementation of old male Ross 308 broiler chickens by EGCG for 4 weeks showed a significant downregulation of the expression of fatty acid synthesis and an upregulation of genes involved in fatty acid β-oxidation and lipolysis. Simultaneously, the activities of fatty acid synthase and acetyl CoA carboxylase were significantly decreased whereas the activity of carnitine palmitoyl transferase-1 was notably elevated by EGCG [82]. To understand the influence of EGCG on fatty acid metabolism, a first study performed on high fat diet mice revealed that EGCG modulates body weight gain through an increase of nuclear respiratory factor (*nrf*) 1, medium chain

acyl CoA decarboxylase (*mcad*), uncoupling protein (*ucp*) 3, and peroxisome proliferator responsive element (*ppar*)-α genes [83]. Furthermore, another *in vitro* study, conducted on human hepatoma HepG2 cells, demonstrated that EGCG inhibited the HMG-CoA lyase activity reducing acetoacetate production and then, prevents ketoacidosis [84]. These studies suggest that EGCG is able to prevent obesity through a modulation involving different organs such as adipose tissue or liver, for example.

6. Involvement of EGCG in Insulin Resistance

Insulin resistance is the key pathophysiological feature of the MS, an important risk factor for cardiovascular disease and diabetes [85]. This pathophysiological condition is defined by a normal insulin concentration that does not adequately produce a normal insulin response in the peripheral target tissues such as adipose, muscle, and liver. The inability of the organism to overcome this insulin resistance leads to hyperinsulinemia, hyperglycemia and type 2 diabetes [86]. If hyperinsulinemia does not allow the maintenance of normoglycemia, it may cause an overexpression of insulin activity in some normally sensitive tissues. In these conditions, the effects of insulin are mediated by an endothelial dysfunction explained in part, by the increased production of endothelin-1 (ET-1) which promotes vasoconstriction, oxidative stress, cell-growth and mitogenesis, and by the activation of the vascular tissue renin–angiotensin system (RAS) [87,88].

Because EGCG has been suggested as a therapeutic agent for the treatment of diabetes, several studies have evaluated the role of this flavonoid in the control of blood glucose concentration. In a study performed on young *db/db* mice fed with diet enriched with EGCG, it has been reported that EGCG improves glucose tolerance and increases glucose-stimulated insulin secretion by preserving islet structure in comparison with control mice [89]. One hypothesis to explain these beneficial effects would be a potentiation of anti-inflammatory properties induced by this flavonoid. In female non-obese diabetic mice treated with 0.05% EGCG in drinking water, a delay of the onset of type 1 diabetes explained by a significant increase of anti-inflammatory cytokine IL-10 has been reported [90]. This hypothesis has been confirmed in another *in vitro* study conducted on RINm5F cells exposed to a combination of recombinant interleukin-1beta (IL-1β), TNF-α, and interferon gamma (IFN-γ), with or without EGCG pretreatment for 24 h. EGCG pretreatment prevented the inflammation-induced destruction of β-cells through a decrease of both mitochondrial reactive-oxygen species production and mitochondrial membrane potential and cytochrome *c* release [91].

In addition to its effects on hyperglycemia, EGCG has also been examined for its effects on diabetes-related comorbidities. Thus, the beneficial effects of this flavonoid have been evaluated in diabetic retinas from Wistar rats and in retinal Müller cells under diabetic conditions. This study revealed that EGCG was able to protect retina against glucose toxicity through an antioxidant mechanism [92]. Furthermore, diabetic nephropathy is one of the most serious complications in diabetes mellitus. Glucose-dependent pathways are activated within the diabetic kidney, such as increasing oxidative stress, polyol formation, and advanced glycation end-products (AGE) accumulation. In a model study of rats in which diabetes has been induced by subtotal nephrectomy and streptozotocin injection, it has been shown that oral administration of EGCG for 50 days suppressed hyperglycemia, proteinuria and lipid peroxidation. Otherwise, it reduced renal advanced glycation end-product accumulation and its related protein expression in the kidney cortex as well as associated pathological conditions [93]. Some recent studies have investigated the properties of EGCG in diabetic neuropathy, the most common complication of diabetes induced by an enhancement of oxidative stress. On streptozotocin-induced diabetic rats, it has been reported that treatment with EGCG for 10 weeks normalized the increase of 8-hydroxy-2′-deoxyguanosine, a marker of oxidative stress, and neuronal hypersensitivity. These findings suggest original properties of EGCG in the prevention of diabetic neuropathy [94].

Among the various mediators involved in the complications of diabetes, osteopontin plays a key role. Osteopontin, a profibrotic adhesion molecule, has been expressed in the renal tubules and glomerular epithelial cells [95]. Although osteopontin is reported to facilitate recovery from acute

tubular injury, it has been shown in renal damage associated with inflammatory glomerulonephritis, obstructive uropathy and tubulointerstitial disease [96]. Based on these findings, osteopontin may be considered as a prognostic marker of diabetic nephropathy. Therefore, a recent study conducted on streptozotocin-induced diabetic nephropathy in mice showed that EGCG 100 mg/kg might provide an effective protection against diabetic nephropathy by osteopontin suppression suggesting that this flavonoid may provide supportive aid for management of *diabetes mellitus* patients with nephropathy [97].

Although the effects of EGCG on type 1 diabetes are interesting, the recent increases in the incidence of obesity make understanding the effects of EGCG against type 2 diabetes very important. As such, in studies conducted on non-obese type 2 diabetic Goto-Kakizaki rats, it has been found that EGCG treatment improved glucose tolerance and glucose homeostasis in GK rats, and reduced oxidative stress and mitochondrial dysfunction in skeletal muscle. These ameliorations have been explained through a down-regulation of the ROS-ERK/JNK-p53 pathway, a reduction of oxidative stress and inhibition of mitochondrial loss and dysfunction [98]. Recently, the direct effects and mechanisms of EGCG on glucose and lipid metabolism have been elucidated in HepG2 cells. Interestingly, it has been reported that EGCG enhanced glycogen synthesis in a dose-dependent manner and inhibited lipogenesis through an enhancement of phosphorylated AMP-activated protein kinase α and acetyl-CoA carboxylase expressions [99]. Otherwise, it has been suggested that EGCG improved insulin sensitivity of HepG2 treated with high glucose, preventing or delaying a potential hepatic dysfunction through the attenuation of the insulin signaling blockade and the modulation of glucose uptake and production. These last findings have been explained by (i) a decrease of tyrosine-phosphorylated and total levels of insulin receptor, insulin receptor substrate (IRS)-1 and -2 triggered by high glucose and (ii) a prevention of the inactivation of the PI3K/AKT pathway and AMPK, as well as a diminution of GLUT-2 levels induced by high glucose [100].

A growing body of evidence indicates that toll-like receptor 4 (TLR4) is a cell surface receptor, a natural immune and pattern recognition receptor expressed in most tissues of the body, that plays a central role in the occurrence of chronic inflammatory diseases, such as obesity-related insulin resistance [101,102]. In a recent study conducted on high-fat diet rats, it has been reported that EGCG significantly decreased free fatty acids, fasting insulin, homeostasis model assessment-insulin resistance index, and epididymal fat coefficient, and increased glucose infusion rate compared to control rats. Furthermore, this study revealed that EGCG attenuated inflammation by decreasing the content of macrophages, interfered the toll-like receptor 4 mediated inflammatory response pathway, thus, improved insulin signaling in adipose tissues [103].

7. Influence of EGCG in Dyslipidemia

Dyslipidemia is characterized by lipids disturbance including an elevation of lipoproteins containing apolipoprotein B (apoB), elevated TGs, increased levels of small particles of LDL, and low levels of HDL- cholesterol. Dyslipidemia, associated with MS, consists of a reduction of HDL-cholesterol and an increase in plasma LDL and TG [104]. As previously described, obesity and insulin resistance play a key role in the development of dyslipidemia associated with MS. Indeed, an elevated lipolysis is observed in the adipose tissue of obese patients, resulting in an important release of FFA and consequently in an increase in TG synthesis and very low density lipoprotein (VLDL) production. Insulin resistance takes part in this process by decreasing ApoB degradation [105,106] and lipoprotein lipase concentration in peripheral tissue that contributes to hypertriglyceridemia and VLDL overproduction [107]. Hypertriglyceridemia, and indirectly insulin resistance, is related to changes in HDL composition and metabolism, leading to an increased clearance of HDL from the circulation. In addition to HDL, the composition of LDL is also modified and patients show a predominance of small dense LDL [108] potentiating the atherogenic risk associated to MS.

Several studies have investigated the relationship between EGCG and the level of blood lipoprotein. Many of them concluded that EGCG is able to reduce total blood cholesterol,

LDL-cholesterol and triglycerides. Thus, from a DNA microarray analysis performed on HepG2 hepatocytes treated with 10 μM or 25 μM EGCG, it has been reported an up-regulation of *ldlr* mRNA and a significant decrease of extracellular apoB levels suggesting beneficial properties of EGCG to improve cholesterol metabolism [109]. Recently, to confirm these first data, the metabolic profile response to administration of EGCG has been studied in high-fat-fed mice. Then, it has been noted that treatment with 50 mg/kg EGCG for 60 days is able to decrease adipose tissue, triglycerides and HDL-cholesterol only in high-fat diet mice [110]. The preventive role of EGCG from hypercholesterolemia has been described in a recent study [111] conducted on Sprague Dawley rats treated with 550 mg/500 mL EGCG. Furthermore, cholesterol and LDL have been reduced by drink containing EGCG in comparison with control drinks. *In vitro* mechanistic studies on EGCG and prevention of dyslipidemia have focused on the antioxidant properties of this polyphenol. Then, it has been reported that EGCG can prevent oxidation of LDL cholesterol *in vitro* [112]. For instance, 1 to 10 g/mL EGCG was shown to dose-dependently reduce LDL oxidation induced by Cu^{2+} [113].

8. Roles of EGCG in Hypertension

Obesity and insulin resistance are now recognized to be associated with hypertension [114,115]. As previously described, these pathophysiological situations are favored by an endothelial dysfunction characterized by an enhancement of RAS mediators expression [116–118] and a decrease in NO bioavailability.

Endothelial dysfunction is characterized by an impaired endothelium-dependent vasodilation inducing a reduced arterial compliance and an increase of inflammation and pro-thrombotic properties [119,120].

The pathophysiology of endothelial dysfunction is complex and involves multiple mechanisms. First of all, reduction of NO availability dependent of oxidative stress is frequently described. Then, NO reacts with O_2^- to form peroxynitrite ($ONOO^-$) [121] a cytotoxic oxidant which alters protein function, oxidizes LDL and leads to a reduced activity of endothelial nitric oxide synthase (eNOS). Besides, ROS upregulated adhesion molecules (ICAM and VCAM) and chemotactic molecules (MCP-1), resulting in establishment of pro-inflammatory state in the vessel wall.

Oxidative stress is intimately linked to inflammation because it may amplify vascular inflammation signaling pathways [122–124].

Obesity, diabetes/insulin resistance, hypertension and MS are known to induce endothelial dysfunction [125–128] which is an important early event in the pathogenesis of atherosclerosis [127] and is consequently a starting point of cardiovascular diseases associated with MS [3].

Thus, endothelial dysfunction is one of the characteristics of hypertension and hypertension is a hallmark of endothelial dysfunction. Therefore, many studies have evaluated the beneficial properties of EGCG to improve endothelial function. One of endothelial dysfunction models is based on the lipid peroxidation induced by asymmetric dimethylarginine (ADMA) [129]. ADMA is synthesized by the protein arginine methyltransferase (PRMT) using *S*-adenosylmethionine as methyl group donor. Conversely, it is degraded by dimethylarginine dimethylamino hydrolase (DDAH), an oxidant-sensitive enzyme with sulfhydryl groups in its structure [130]. ADMA and DDAH are widely distributed in endothelial cells [131] and ADMA is thought to induce endothelial dysfunction through an inhibition of eNOS by competing with L-arginine [132]. Thus, in HUVEC treated with 100 μg/mL oxidized low density lipoprotein (ox-LDL), EGCG (10 and 100 mg/mL) significantly increased the level of nitrite/nitrate and the activity of DDAH suggesting that EGCG improved endothelial dysfunction by decreasing level of ADMA and by enhancing endothelial nitric oxide production. Moreover, in the same study, in a model of endothelial dysfunction induced by LDL in rats, it has been confirmed that EGCG (10 or 50 mg/kg) significantly attenuated the inhibition of vasodilator response to acetylcholine through a decreased serum nitrite/nitrate level associated with a decrease of the elevated levels of ADMA [133].

Furthermore, to understand the structure-activity relationship causing increased production of NO, a recent study has examined the effect of selective replacement of hydroxyl functions on either the B or D ring on the EGCG-induced phosphorylation of AKT and eNOS, formation of ROS and NO in cultured coronary artery endothelial cells, and endothelium-dependent relaxation of coronary artery rings. Interestingly, it has been found that the hydroxyl group at the 3′ position of the gallate ring is essential and, also, to some extent, the two hydroxyl groups at positions 3′ and 4′, for the PI3-kinase/AKT-dependent phosphorylation of endothelial NO synthase leading to the subsequent NO-mediated vascular relaxation [134].

Other pathophysiological mechanisms may explain hypertension. Thus, the kidneys increase sodium reabsorption, the heart increases cardiac output, and arteries respond with vasoconstriction resulting in hypertension. Secondly, compression exerted by the visceral fat on the renal parenchyma may cause hemodynamic disturbances [135]. Finally, adipocytes are able to produce aldosterone in response to angiotensin II and may be considered as a miniature renin-angiotensin-aldosterone system [136]. All this mechanisms may contribute to the development of hypertension in patients with insulin resistance and/or obesity.

As previously shown, the RAS plays a major role in regulating blood pressure in animals [137], and renin is a crucial enzyme whose inhibition is considered as a useful approach to treat hypertension. Few studies have analyzed the inhibitory effects of EGCG on renin activity. However, in a recent *in vitro* study, it has been reported inhibitory properties of EGCG with an inhibitory concentration 50 (IC_{50}) value of 44.53 μM. Furthermore, this study revealed that EGCG acted in an uncompetitive manner and suggested that galloyl moiety and ortho-trihydroxy phenyl structures might be favorable for the renin-inhibitory activity of EGCG [138]. The beneficial properties of EGCG have been examined on spontaneously hypertensive rats (SHR), a model of hypertension, insulin resistance and obesity. In a study conducted by Potenza *et al.*, it has been showed a significant decrease of blood pressure equivalent in rats treated with 3 mg/kg/day enalapril (an angiotensin converting enzyme inhibitor) and rats treated with 200 mg/kg/day EGCG compared to SHR control. Additionally, this study confirmed that EGCG stimulated nitric oxide production from endothelium through a PI-3-kinase pathway suggesting that EGCG may be relevant to improve symptoms of metabolic syndrome and particularly, hypertension [139].

9. Conclusions and Perspectives

There is traditional and widespread use of dietary flavonoids all around the world. While anecdotal and epidemiological evidence has historically supported the idea of a link between varied diet and good health, experimental evidence supports the idea that dietary flavonoids have potentially beneficial effects on a multitude of health conditions, including metabolic syndrome. As discussed in this review, the beneficial properties of EGCG have been established in both various cell lines and different animal models. Studies in cell lines have also demonstrated that these compounds can affect a range of signaling and metabolic pathways resulting in improving various symptoms including endothelial dysfunction.

In recent years, evidence has suggested that DNA methylation is involved in the emergence of metabolic syndrome through the epigenetic regulation of numerous candidate genes. Thus, a particular attention has been focused on epigenetic modulations induced by obesity. In fact, cell studies showed methylation variations in genes involved in energy metabolism such as *ppar-α*, *ucp1* and *phosphoenolpyruvate carboxinase* [140]. Furthermore, the genes of *leptin receptor* and *leptin* have been found to be mutated in obese individuals [141]. Hypertension, another symptom of metabolic syndrome, showed variations in DNA modulations since it has been reported in hypertensive rat models a hypomethylation of the *(pro)renin* gene [142] or of the *adrenergic β1* gene [143]. Interestingly, recent studies have described the beneficial properties of flavonoids to prevent obesity or hypertension through a regulation of DNA methylation patterns [144,145]. Regarding EGCG, the most existing studies have focused on the modulation of DNA methylation in tumorigenesis suggesting interesting

scientific opportunities to determine the properties of EGCG in DNA methylation, particularly in metabolic syndrome.

On the basis of these results, one can advance the notion that EGCG is readily available and widely consumed and may have a high potential use in the prevention of metabolic syndrome. Nevertheless, the preventive activity of this compound has not been consistently observed in human studies. Although some clinical studies have evaluated the preventive properties of EGCG in obesity (Table 3), other clinical studies should be considered in order to provide conclusions about the use of EGCG to prevent all the symptoms of the metabolic syndrome.

Table 3. Properties of EGCG on human obesity.

Subjects	Dose	Duration	Results	Ref
115 obese women		12 weeks	↓ body weight ↓ BMI ↓ total cholesterol ↓ LDL cholesterol	[146]
56 obese, hypertensive patients	379 mg/day	12 weeks	↓ SBP, ↓ DBP ↓ serum glucose ↓ insulin resistance ↓ LDL cholesterol ↓ TG	[147]
46 obese patients	379 mg/day	12 weeks	↓ BMI ↓ body weight ↓ serum glucose ↓ total cholesterol ↓ LDL cholesterol ↓ TG	[148]
35 obese patients with MS	870 mg/day	8 weeks	↓ body weight ↓ BMI ↓ LDL cholesterol ↓ LDL/HDL ratio	[149]
88 obese patients	800 mg/day	8 weeks	↓ DBP	[150]
40 obese children	576 mg/day	24 weeks	↓ body weight ↓ SBP ↓ LDL cholesterol	[151]

BMI: body mass index; LDL: low density lipoprotein; SBP: systolic blood pressure; DBP: diastolic blood pressure; MS: metabolic syndrome; TG: triglycerides.

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Relationships of Dietary Histidine and Obesity in Northern Chinese Adults, an Internet-Based Cross-Sectional Study

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Abstract: Our previous studies have demonstrated that histidine supplementation significantly ameliorates inflammation and oxidative stress in obese women and high-fat diet-induced obese rats. However, the effects of dietary histidine on general population are not known. The objective of this Internet-based cross-sectional study was to evaluate the associations between dietary histidine and prevalence of overweight/obesity and abdominal obesity in northern Chinese population. A total of 2376 participants were randomly recruited and asked to finish our Internet-based dietary questionnaire for the Chinese (IDQC). Afterwards, 88 overweight/obese participants were randomly selected to explore the possible mechanism. Compared with healthy controls, dietary histidine was significantly lower in overweight ($p < 0.05$) and obese ($p < 0.01$) participants of both sexes. Dietary histidine was inversely associated with body mass index (BMI), waist circumference (WC) and blood pressure in overall population and stronger associations were observed in women and overweight/obese participants. Higher dietary histidine was associated with lower prevalence of overweight/obesity and abdominal obesity, especially in women. Further studies indicated that higher dietary histidine was associated with lower fasting blood glucose (FBG), homeostasis model assessment of insulin resistance (HOMA-IR), 2-h postprandial glucose (2 h-PG), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), C-reactive protein (CRP), malonaldehyde (MDA) and vaspin and higher glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and adiponectin of overweight/obese individuals of both sexes. In conclusion, higher dietary histidine is inversely associated with energy intake, status of insulin resistance, inflammation and oxidative stress in overweight/obese participants and lower prevalence of overweight/obesity in northern Chinese adults.

Keywords: dietary histidine; overweight/obesity; insulin resistance; inflammation; oxidative stress

1. Introduction

The rising prevalence of overweight and obesity has become a major global health challenge [1]. Obesity is a major risk factor for a series of metabolic disorders and chronic diseases, including insulin resistance, metabolic syndrome, type 2 diabetes, hypertension, cardiovascular diseases and certain

cancer [2–5]. Accumulating evidence has proved that nutritional factors are strongly associated with the development, treatment and prevention of chronic diseases, such as obesity, hyperlipidemia, diabetes mellitus and cardiovascular diseases [6–9]. Therefore, it is urgent to improve etiologic research on diet by investigating nutrient and food intakes and reasonable dietary guidelines should be proposed by the government.

Histidine, a precursor for the synthesis of histamine, is abundant in red meat and fish and is an important amino acid for humans. It has been reported that lower plasma concentration of histidine is associated with protein-energy wasting, inflammation and oxidative stress in chronic kidney disease patients [10]. Previous animal studies confirmed that histidine supplementation could reduce body weight and ameliorates inflammation and oxidative stress of adipose tissue in a high-fat diet induced female obese rat model [11]. Moreover, dietary histidine also suppresses food intake and fat accumulation in rats [12]. In our previous study, we had found that serum histidine concentrations in obese women were significantly lower than those in non-obese women and had negative relationships with inflammation and oxidative stress [13]. The recovery of serum histidine concentrations through histidine supplementation could improve insulin resistance, reduce body mass index (BMI) and fat mass, and suppress inflammation and oxidative stress in obese women [14]. Only one cross-sectional study in Japanese adolescents showed a significantly negative correlation between energy intake and ratio of histidine to protein intake [15].

Although the central roles of dietary histidine for regulation of energy intake have already been established in animal and in vitro studies, to our knowledge, no study has analyzed the associations between dietary histidine and prevalence of obesity in general population. Moreover, whether chronic intake of dietary histidine is associated with insulin resistance, inflammation and oxidative stress in overweight/obese individuals is still unclear. The contribution of dietary histidine is not yet fully confirmed. Therefore, we assessed dietary histidine intakes of 2376 participants and examined relationships between dietary histidine and obesity in this population in this study. Furthermore, a subgroup study of 88 overweight/obese subjects (44 men and 44 women) was performed to explore the possible mechanism of the anti-obesity effect of histidine.

2. Materials and Methods

2.1. Development and Validation of the IDQC

To investigate dietary habits accurately, many methods for dietary assessment have been used by researchers. The food frequency questionnaire (FFQ) provides us a convenient and economic method to assess the general dietary intakes in a large population [16]. However, traditional face-to-face FFQs are impossible to conduct in a large population during a short period as the face-to-face FFQs always consume much time [17]. Previously, a convenient tool named Internet-based dietary questionnaire for the Chinese (IDQC) has been designed and validated at Harbin Medical University previously by experts of nutrition, epidemiology and bio-statistics [18].

Commonly eaten foods were divided into 16 categories (i.e., grains, potatoes, legumes, vegetables, fungus, fruits, seeds and nuts, livestock, poultry, dairy, eggs, fish, snacks, sugar, condiments, and beverages). Reference images of each food item's weight/volume were created as references to assist the participants in making accurate estimation of food portions. Each participant had to choose the frequency and amount of each subtype of food groups. Finally, the questionnaire was uploaded to a secure website, which is free of access [19].

The IDQC has been validated as a convenient dietary assessment tool in our previous study [18]. Briefly, 644 recruited participants had completed the IDQC, the intakes of the food groups and nutrients in the IDQC were validated against those in the 3-day dietary records. The IDQC has been confirmed as an accurate tool for dietary assessment, because it has good consistency to results of 3-day dietary records. Therefore, the IDQC can be used for dietary assessment in large population.

2.2. Participants, Exclusive Criteria, Power Calculation and Study Design

We randomly invited 3626 participants from the Health Examination Center of the Second Affiliated Hospital of Harbin Medical University. Of the 3626 invited participants, 2995 agreed to participate in our study, register an account and finish the IDQC. Firstly, online demographic questionnaires were completed by all participants; the details of which were provided in our previous published manuscript [20]. The exclusive criteria were as follows: (1) incomplete information on the IDQC; (2) extreme daily energy intake (<800 kcal (3349 kJ) or >5000 kcal (20,934 kJ) for males; <600 kcal (2512 kJ) or >4000 kcal (16,747 kJ) for females); (3) low BMI (BMI < 18.5); and (4) diabetes diagnosis or being on a diet in the past 6 months. In total, 619 were excluded for the above reasons (Figure 1).

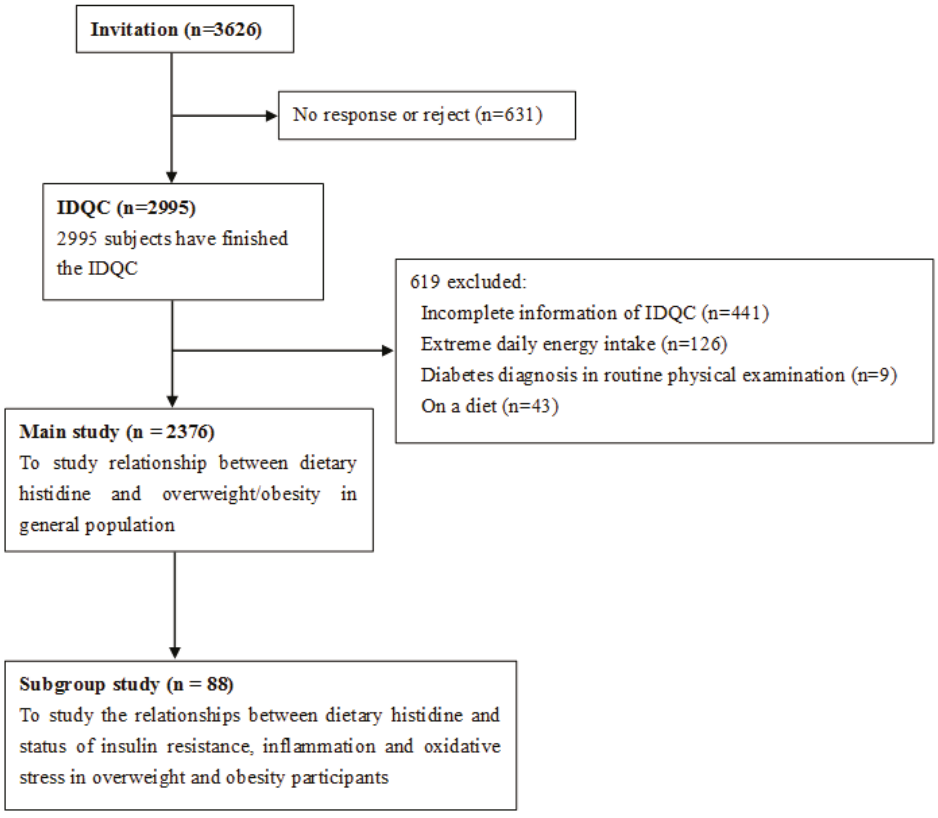

Figure 1. Flow of the study population.

For power calculation, considering the 9.9 cm standard deviation of waist circumference (WC) in the overall population, a sample size of 594 in each quartile will be sufficient to detect a difference of 2.4 cm between quartiles. Moreover, this sample size is also sufficient to detect a difference of 1.0 kg/m^2 in BMI, at 99% power and 5% level of significance.

To explore possible mechanisms of the anti-obesity effect of histidine, 88 overweight/obese subjects were randomly selected by stratified sampling for subgroup study. Except for dietary histidine (% protein), body weight, BMI, WC, and drinking and smoking habits, there is no difference between included participants and excluded participants. Characteristics are presented in Table S1. An oral glucose tolerance test (OGTT) was performed and metabolic profile, status of inflammation and

oxidative stress were also examined. A flow chart of the study population is shown in Figure 1. This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving humans were approved by the Human Research Ethics Committee of the Harbin Medical University (approval code [2015]006). Online informed consent was obtained from all participants.

2.3. Estimation of Dietary Nutrients Intake

After the online demographic questionnaire, all participants were asked to complete the IDQC for the past 4 months, and details of the IDQC are provided in our previous published manuscript [20]. The intake detail (frequency and amount) of each kind of food was obtained and average daily intakes of all nutrients were then calculated according to the China Food Composition Tables [21]. China Food Composition Tables is an important reference book for researchers of nutrition and public health of China. In this book, commonly eaten Chinese foods are evaluated and average nutrient contents (including energy, macro nutrients, trace elements, amino acids, and fatty acids) of these foods are measured by researchers and provided in the book. In our study, the histidine amount is total dietary source histidine (including protein, peptide and free form of histidine).

2.4. Anthropometric Measurements

All participants were asked to stand on an anthropometer without heavy clothing and shoes. Body weight and height were measured to the nearest 0.1 kg and 0.1 cm. For WC measurement, the same standard was applied for each individual. WC was measured using a flexible anthropometric tape on the horizontal plane between the lowest rib and the iliac crest, to the nearest 0.1 cm. Blood pressure was measured using a standard mercury sphygmomanometer. Each participant was seated comfortably for 10 min in a quiet room. Korotkoff sounds I and V were criteria for systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively. SBP and DBP were measured twice and the mean values were calculated. All anthropometric measurements were performed by standardized and trained personnel and have good reproducibility.

2.5. Definition of Overweight/Obesity and Abdominal Obesity

BMI was defined as weight in kilograms divided by the square of height in meters (kg/m^2). As this study was performed in Chinese population, the BMI cut-off points of Chinese subjects (overweight: 24.0–27.9; obesity $\geqslant$ 28.0) were used [22]. Abdominal obesity was defined as WC $\geqslant$ 85 cm in men and WC $\geqslant$ 80 cm in women, according to the 2006 Guidelines on Preservation and Control Overweight and Obesity in Chinese Adults classification [23].

2.6. Collection and Laboratory Analysis of Serum

Eighty-eight participants were instructed to fast overnight (more than 12 h) and fasting blood samples were collected in the morning. After fasting blood samples were obtained, each participant was asked to take 75 g glucose (dissolved in 200 mL of warm water), and after 2 h, postprandial blood samples were obtained. Blood samples were centrifuged at 3000 rpm for 15 min to obtain serum and serum were stored at $-80\ ^{\circ}C$ immediately. Fasting and 2 h-postprandial blood glucose levels were measured with a hand-held glucose monitoring system (One-Touch Ultra 2; Life Scan, Milpitas, CA, USA). Serum insulin concentration was measured by ROCHE Elecsys 2010 Chemiluminescence Immune Analyzer (Roche Diagnostics, Mannheim, Germany). Fasting and 2 h-postprandial serum triglyceride (TG,) total cholesterol (TC), high destiny lipoprotein (HDL) and low destiny protein (LDL) were determined using a ROCHE Modular P800 Automatic Biochemical Analyzer (Roche Diagnostics, Mannheim, Germany). Homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as previously described [24]. Serum tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), C-reactive protein (CRP), adiponectin and vaspin concentrations were assayed using ELISA with commercial kits (TNF-α, IL-1β, IL-6, and vaspin, catalog number CSB-E04740h, CSB-E08053h, CSB-E04638h, CSB-E09771h, Cusabio, China; CRP, catalog number BC-1119, Biocheck,

USA; adiponectin, catalog number ab99968, Abcam, UK). Serum superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malonaldehyde (MDA) were measured with enzymatic methods using commercial kits (Jiancheng Technology, Nanjing, China). Coefficients of variations (CVs) of lab measurements are presented in Supplementary Materials.

2.7. Statistical Analysis

For descriptive statistics, means and SDs (or frequencies and percentages) were calculated across BMI categories (normal weight: BMI < 24.0; overweight: 24.0–27.9; obese: ⩾28.0) and compared using one-way analysis of variance (one-way ANOVA) or chi-square test, as appropriate. Dietary histidine of different BMI categories was further analyzed by means of analysis of covariance (ANCOVA).

Considering the high correlation between histidine and total protein intake ($r = 0.925$), and that nutrient densities can be used to reduce the likelihood of multicollinearity [25], we used histidine (percent total protein intake) in our study. Bivariate correlation analysis (without variants adjusted) and partial correlation analysis was performed to assess the association of branched-chain amino acid (BCAA) and BMI, WC, SBP and DBP (adjusting for age, dietary carbohydrate, fat, protein, cholesterol and fiber intake), fasting blood glucose (FBG), insulin, HOMA-IR, TC, TG, HDL, LDL, 2-h postprandial glucose (2 h-PG), 2 h-insulin, 2 h-TC, 2 h-TG, 2 h-HDL, 2 h-LDL, GSH-Px, SOD, MDA, TNF-α, IL-1β, IL-6, CRP, adiponectin and vaspin (adjusting for age, BMI, dietary carbohydrate, fat, protein, cholesterol and fiber intake).

To estimate the Odds ratio (OR) and 95% confidence interval (CI) of overweight/obesity and abdominal obesity between quartiles of dietary histidine, logistic regression model was used. Age, education, income, labor, exercise status, dietary carbohydrate, fat, protein, cholesterol, fiber intake and smoking habits in three models. The statistical analyses were carried out using SAS software (version 9.1; SAS Institute, Cary, NC, USA). By statistician and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Descriptive Statistics

The characteristics of participants by dietary histidine quartiles are summarized in Table 1. Compared with those in the first quartile of dietary histidine, participants of the 4th quartile were more likely to be youthful and had lower body weight, BMI, WC, and higher percentage of women. The highest prevalence of current smoking was observed in the 2nd quartile of dietary histidine. Income, education, labor and physical exercise status were also different between histidine quartiles (all $p < 0.05$). SBP and DBP of the 4th quartile were also significantly lower than those in the 1st quartile of dietary histidine. The prevalence of overweight/obesity was 37.4% and the prevalence of abdominal obesity was 28.5% among our participants.

For dietary intake, total protein, total amino acids, total fat, cholesterol and fiber of the 3rd and 4th quartiles were significantly higher than the 1st quartile. Dietary carbohydrates of the 1st quartile was significantly higher than the 3rd and 4th quartiles. Total energy intake was also different between histidine quartiles ($p = 0.048$).

Table 1. Characteristics and dietary intake of participants by quartile of histidine intake.

	Quartiles of Histidine (% Total Protein Intake)				*p*
	1	**2**	**3**	**4**	
Histidine, % total protein intake	<1.38	1.38–1.59	1.59–1.77	>1.77	
Participants, *n*	594	594	594	594	
BMI categories					<0.001
<24.0, *n* (%)	327 (55.1)	352 (59.3)	384 (64.6)	424 (71.4)	
24.0–28.0, *n* (%)	191 (32.2)	182 (30.6)	165 (27.8)	141 (23.7)	
⩾28.0, *n* (%)	76 (12.8)	60 (10.1)	45 (7.6)	29 (4.9)	
Abdominal obesity					0.020
Yes, *n* (%)	191 (32.1)	174 (29.3)	168 (28.3)	143 (24.1)	
No, *n* (%)	403 (67.9)	420 (70.7)	426 (71.7)	451 (75.9)	
Age, year	34.7 ± 14.9	34.6 ± 16.3	33.3 ± 15.5	29.9 ± 14.5	<0.001
Gender					0.018
Men, *n* (%)	319 (53.7)	296 (49.8)	266 (44.8)	283 (47.6)	
Women, *n* (%)	275 (46.3)	298 (50.2)	328 (55.2)	311 (52.4)	
Body weight, kg	67.6 ± 12.5	66.1 ± 11.6	65.4 ± 11.3	64.2 ± 11.4	<0.001
BMI, kg/m^2	23.8 ± 3.7	23.3 ± 3.3	23.2 ± 3.3	22.8 ± 3.1	<0.001
WC, cm	80.6 ± 10.3	79.2 ± 9.7	79.0 ± 9.5	78.2 ± 9.8	<0.001
Income per month					<0.001
<2000 yuan, *n* (%)	355 (59.8)	366 (61.6)	374 (63.0)	442 (74.4)	
2000–5000 yuan, *n* (%)	223 (37.5)	207 (34.8)	203 (34.2)	123 (20.7)	
⩾5000 yuan, *n* (%)	16 (2.7)	21 (3.5)	17 (2.9)	29 (4.9)	
Education					0.021
Under college, *n* (%)	175 (29.5)	145 (24.4)	132 (22.2)	122 (20.5)	
Bachelor, *n* (%)	398 (67.0)	424 (71.4)	437 (73.6)	449 (75.6)	
Master or doctor, *n* (%)	21 (3.5)	25 (4.2)	25 (4.2)	23 (3.9)	
Labor					<0.001
Light, *n* (%)	164 (27.6)	179 (30.1)	149 (25.1)	135 (22.7)	
Medium, *n* (%)	391 (65.8)	397 (66.8)	430 (72.4)	453 (76.3)	
Heavy, *n* (%)	39 (6.6)	18 (3.0)	15 (2.5)	6 (1.0)	
Exercise					<0.001
<10 h/week, *n* (%)	281 (47.3)	223 (3.8)	182 (30.6)	137 (23.1)	
10–20 h/week, *n* (%)	300 (50.5)	315 (53.0)	317 (53.4)	334 (56.2)	
⩾20 h/week, *n* (%)	13 (2.2)	56 (9.4)	95 (16.0)	123 (20.7)	
Smoking					0.046
Non-smoker, *n* (%)	493 (83.0)	494 (83.2)	530 (89.2)	507 (85.4)	
Current smoker, *n* (%)	73 (12.3)	75 (12.6)	49 (8.2)	61 (10.3)	
Quit smoking, *n* (%)	28 (4.7)	25 (4.2)	15 (2.5)	26 (4.4)	
Drinking					0.44
Non-drinker, *n* (%)	475 (80.0)	489 (82.3)	495 (83.7)	480 (80.8)	
Current drinker, *n* (%)	119 (20.0)	105 (17.7)	99 (16.7)	114 (19.2)	
SBP (mmHg)	119.1 ± 12.9	119.0 ± 14.1	117.7 ± 12.4	115.1 ± 11.4	<0.001
DBP (mmHg)	78.9 ± 8.7	78.7 ± 9.1	77.9 ± 8.9	76.2 ± 8.4	<0.001
Dietary intakes					
Energy, kcal/day	2448.4 ± 824.5	2534.2 ± 867.6	2430.5 ± 865.5	2399.9 ± 896.0	0.048
Total protein, g/day	79.7 ± 30.5	92.7 ± 35.2	93.8 ± 37.9	95.0 ± 41.7	<0.001
Total amino acids, g/day	36.9 ± 16.8	54.2 ± 20.7	61.3 ± 24.7	69.8 ± 30.5	<0.001
Histidine, g/day	0.9 ± 0.4	1.4 ± 0.5	1.6 ± 0.6	1.8 ± 0.8	<0.001
Total fat, g/day	60.8 ± 28.2	78.5 ± 35.3	83.4 ± 37.6	81.2 ± 43.0	<0.001
Total carbohydrate, g/day	406.9 ± 146.3	382.5 ± 137.9	345.2 ± 133.4	341.9 ± 141.9	<0.001
Cholesterol, mg/day	405.7 ± 309.9	495.8 ± 338.9	489.6 ± 309.6	435.8 ± 349.0	<0.001
Fiber, g/day	15.7 ± 7.6	20.9 ± 10.6	21.7 ± 11.1	22.6 ± 13.0	<0.001

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; *p* are for differences across quartiles of histidine intake. Data are expressed as mean ± SD or frequencies and percentages, as appropriate.

3.2. Dietary Histidine of Overweight and Obesity Participants Was Lower than Healthy Controls

We firstly performed this comparison to find the differences in dietary histidine among three BMI categories (normal weight < 24.0, overweight 24.0–28.0, and obesity ⩾ 28.0). In the overall male and female populations, dietary histidine of overweight and obese groups were all significantly lower than normal group ($p < 0.05$ between normal and overweight group, $p < 0.01$ between normal and obese group). Moreover, dietary histidine of obese group was significantly lower than overweight group ($p < 0.05$). Confounding factors such as age, education, income, labor, exercise status, dietary carbohydrate, fat, protein, fiber and cholesterol intake and smoking habits were controlled in this study (Figure 2).

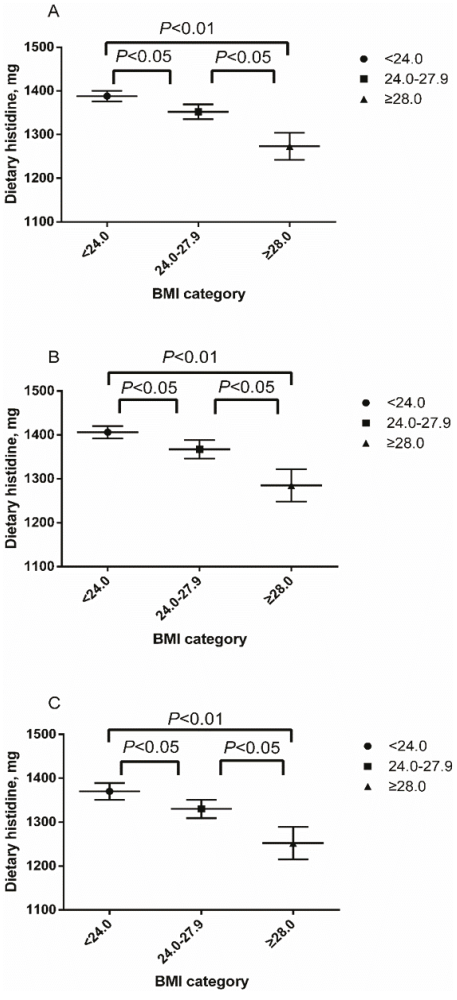

Figure 2. Dietary histidine of different body mass index (BMI) categories: (**A**) the overall population; (**B**) male participants; and (**C**) female participants. Confounding factors such as age, labor, exercise status, dietary carbohydrate, fat, protein, fiber and cholesterol intake and smoking habits were controlled.

3.3. Correlations between Dietary Histidine and Body Weight, BMI, WC, SBP and DBP

Bivariate correlation analysis and partial correlation analysis was used to examine these associations of dietary histidine with body weight, BMI, WC, SBP and DBP. Potential covariates (age, dietary carbohydrate, fat, protein, fiber and cholesterol intake) were adjusted in the partial correlation analysis. As shown in Table 2, we found negative correlations between dietary histidine and body weight, BMI, WC, SBP and DBP ($r = -0.076$, -0.129, -0.132, -0.106 and -0.092, respectively, all $p < 0.01$). Because of higher histidine intake in males (male: 1.6 ± 0.8 g vs. female: 1.3 ± 0.6 g, $p < 0.01$), we examined these associations in both gender, the significance was stronger in women. Furthermore, considering the higher histidine intake in normal BMI participants (Figure 2), we examined these associations in different BMI categories. We found negative correlations between dietary histidine and BMI, WC, SBP and DBP in overweight and obese participants, but no significant correlation of dietary histidine and BMI, WC, and DBP were observed in normal BMI participants.

Table 2. Correlations between dietary histidine and energy intake, body weight, BMI, WC and blood pressure.

	Unadjusted		**Adjusted** *			**Unadjusted**		**Adjusted** *	
	r	***p***	***r***	***p***		***r***	***p***	***r***	***p***
Overall					Normal BMI				
Energy intake	−0.031	NS	−0.053	<0.05	Energy intake	−0.032	NS	−0.027	NS
Body weight	−0.093	<0.001	−0.076	<0.01	Body weight	−0.042	NS	−0.029	NS
BMI	−0.126	<0.001	−0.129	<0.001	BMI	−0.046	NS	−0.037	NS
WC	−0.127	<0.001	−0.132	<0.001	WC	−0.061	NS	−0.071	NS
SBP	−0.119	<0.001	−0.106	<0.001	SBP	−0.109	<0.001	−0.056	<0.05
DBP	−0.104	<0.001	−0.092	<0.001	DBP	−0.079	<0.01	−0.052	NS
Men					Overweight				
Energy intake	−0.022	NS	−0.034	NS	Energy intake	−0.055	NS	−0.058	NS
Body weight	−0.061	<0.05	−0.064	<0.05	Body weight	−0.094	NS	−0.042	NS
BMI	−0.102	<0.01	−0.095	<0.05	BMI	−0.137	<0.001	−0.133	<0.001
WC	−0.096	<0.05	−0.093	<0.05	WC	−0.114	<0.05	−0.125	<0.05
SBP	−0.143	<0.001	−0.076	<0.05	SBP	−0.104	<0.01	−0.097	<0.05
DBP	−0.112	<0.001	−0.086	<0.01	DBP	−0.112	<0.01	−0.092	<0.05
Women					Obesity				
Energy intake	−0.065	<0.05	−0.056	<0.05	Energy intake	−0.068	<0.05	−0.073	<0.05
Body weight	−0.096	<0.05	−0.088	<0.01	Body weight	−0.119	<0.05	−0.104	<0.05
BMI	−0.136	<0.001	−0.147	<0.001	BMI	−0.131	<0.01	−0.147	<0.001
WC	−0.138	<0.01	−0.153	<0.001	WC	−0.143	<0.01	−0.142	<0.01
SBP	−0.066	<0.05	0.113	<0.01	SBP	−0.117	<0.01	−0.113	<0.05
DBP	−0.065	<0.05	−0.109	<0.01	DBP	−0.143	<0.05	−0.131	<0.05

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, no significance. * Adjusting for dietary carbohydrate, fat, protein, fiber and cholesterol intake.

3.4. Higher Dietary Histidine Intake Is Associated with Lower Prevalence of Overweight/Obesity and Abdominal Obesity in Northern Chinese

With adjustment for potential dietary and non-dietary cofounders (age, gender; income; education; labor; physical exercise; dietary carbohydrate, fat, protein, fiber and cholesterol intake; and smoking status), higher dietary histidine was inversely associated with prevalence of overweight/obesity and Abdominal obesity. As shown in Tables 3 and 4, compared with the 1st quartile, the multivariable-adjusted ORs of overweight/obesity for the 3rd and 4th quartile were 0.745 (0.572, 0.969) and 0.650 (0.482, 0.876), respectively (all $p < 0.05$). After being stratified by gender, the significance still exists in the 4th quartile of men and the 2nd, 3rd and 4th quartile of women (all $p < 0.05$). For abdominal obesity, the multivariable-adjusted ORs for the 2nd, 3rd and 4th quartile were 0.716 (0.539, 0.952), 0.809 (0.597, 0.995) and 0.754 (0.545, 0.943), respectively (all $p < 0.05$). After being stratified by gender, the significance is stronger in women (all $p < 0.01$) but no significant correlation was observed in men.

Table 3. Multivariable-adjusted Odds ratio (OR) and 95% confidence interval (CI) of overweight/obesity by quartile of dietary histidine.

All				
Histidine Quartiles	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**
Dietary histidine (% protein)	<1.38	1.38–1.59	1.59–1.77	>1.77
Participants, *n*	594	594	594	594
Overweight/obesity, *n* (%)	267 (44.9)	242 (40.7)	210 (35.4)	170 (28.6)
Crude	1	0.768 (0.610, 0.968) *	0.736 (0.584, 0.928) *	0.537 (0.423, 0.681) **
Model 1	1	0.772 (0.602, 0.990) *	0.752 (0.586, 0.966) *	0.646 (0.500, 0.834) **
Model 2	1	0.777 (0.587, 1.029)	0.744 (0.572, 0.968) *	0.649 (0.481, 0.875) **
Model 3	1	0.772 (0.583, 1.023)	0.745 (0.572, 0.969) *	0.650 (0.482, 0.876) **
Men				
Histidine Quartiles	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**
Dietary histidine (% protein)	<1.35	1.35–1.57	1.57–1.76	>1.76
Participants, *n*	291	291	291	291
Overweight/obesity, *n* (%)	132 (45.4)	117 (40.2)	98 (33.7)	85 (29.2)
Crude	1	0.926 (0.671, 1.278)	0.826 (0.592, 1.154)	0.608 (0.434, 0.853) **
Model 1	1	0.919 (0.655, 1.289)	0.843 (0.594, 1.197)	0.599 (0.490, 0.998) *
Model 2	1	0.973 (0.676, 1.388)	0.828 (0.561, 1.288)	0.578 (0.468, 0.991) *
Model 3	1	0.969 (0.672, 1.396)	0.838 (0.577, 1.302)	0.575 (0.456, 0.988) *
Women				
Histidine Quartiles	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**
Dietary histidine (% protein)	<1.40	1.40–1.61	1.61–1.77	>1.77
Participants, *n*	303	303	303	303
Overweight/obesity, *n* (%)	135 (44.6)	125 (41.3)	112 (37.0)	85 (28.1)
Crude	1	0.639 (0.461, 0.884) **	0.624 (0.448, 0.871) **	0.463 (0.330, 0.649) **
Model 1	1	0.642 (0.445, 0.925) *	0.574 (0.393, 0.837) **	0.549 (0.376, 0.803) **
Model 2	1	0.607 (0.403, 0.915) *	0.557 (0.375, 0.827) **	0.515 (0.333, 0.796) **
Model 3	1	0.612 (0.406, 0.923) *	0.556 (0.374, 0.826) **	0.518 (0.335, 0.801) **

Model 1: adjusting for age, education, income, labor and exercise status. Model 2: Model 1 + dietary carbohydrate, fat, protein, fiber and cholesterol intake were adjusted. Model 3: Model 2 + smoking habits were adjusted. * $p < 0.05$; ** $p < 0.01$ compared with the 1st quartile.

3.5. Correlations between Dietary Histidine and Status of Insulin Resistance, Inflammation and Oxidative Stress in Overweight/Obese Participants

Considering the stronger correlations between dietary histidine and BMI and WC in overweight/obese participants, 88 overweight/obese individuals were selected for subgroup study to explore the possible mechanism. Characteristics of subgroup study population are provided in Table S1. Overweight and obesity are always accompanied by abnormal postprandial metabolism, chronic low-grade inflammation and oxidative stress [20]. Therefore, serum biochemistry indexes and biomarkers of inflammation and oxidative stress were measured. Partial correlation analysis was used to examine these associations of dietary histidine with status of insulin resistance, inflammation and oxidative stress, independent of total energy intake. Potential covariates (age, BMI, dietary carbohydrate, fat, protein, fiber and cholesterol intake) were adjusted in the model. As shown in Table 5, higher dietary histidine is associated with lower FBG, HOMA-IR, 2 h-PG, MDA, TNF-α, IL-1β, IL-6, CRP, MDA and vaspin and higher GSH-Px, SOD and adiponectin of overweight/obese individuals of both sexes (all $p < 0.05$).

Table 4. Multivariable-adjusted Odds ratio (OR) and 95% confidence interval (CI) of abdominal obesity by quartile of dietary histidine.

All				
Histidine Quartiles	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**
Dietary histidine (% protein)	<1.38	1.38–1.59	1.59–1.77	>1.77
Participants, *n*	594	594	594	594
Abdominal obesity, *n* (%)	191 (32.1)	174 (29.3)	168 (28.3)	143 (24.1)
Crude	1	0.771 (0.603, 0.987) *	0.752 (0.587, 0.963) *	0.614 (0.477, 0.792) **
Model 1	1	0.733 (0.563, 0.956) *	0.774 (0.594, 0.996) *	0.742 (0.565, 0.974) *
Model 2	1	0.723 (0.545, 0.059) *	0.782 (0.579, 0.998) *	0.761 (0.552, 0.985) *
Model 3	1	0.716 (0.539, 0.952) *	0.809 (0.597, 0.995) *	0.754 (0.545, 0.943) *
Men				
Histidine Quartiles	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**
Dietary histidine (% protein)	<1.35	1.35–1.57	1.57–1.76	>1.76
Participants, *n*	291	291	291	291
Abdominal obesity, *n* (%)	120 (41.2)	118 (40.5)	114 (39.2)	102 (35.1)
Crude	1	0.972 (0.698, 1.353)	0.918 (0.659, 1.279)	0.769 (0.550, 1.075)
Model 1	1	0.943 (0.663, 1.341)	0.923 (0.648, 1.315)	0.908 (0.634, 1.301)
Model 2	1	0.948 (0.650, 1.383)	0.940 (0.627, 1.410)	0.928 (0.600, 1.435)
Model 3	1	0.929 (0.636, 1.357)	0.959 (0.639, 1.441)	0.916 (0.592, 1.419)
Women				
Histidine Quartiles	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**
Dietary histidine (% protein)	<1.40	1.40–1.61	1.61–1.77	>1.77
Participants, *n*	303	303	303	303
Abdominal obesity, *n* (%)	71 (23.4)	56 (18.5)	54 (17.8)	41 (13.5)
Crude	1	0.639 (0.461, 0.884) **	0.624 (0.448, 0.871) **	0.463 (0.330, 0.649) **
Model 1	1	0.499 (0.313, 0.794) **	0.539 (0.280, 0.938) *	0.507 (0.314, 0.816) **
Model 2	1	0.477 (0.293, 0.777) **	0.476 (0.278, 0.802) **	0.472 (0.272, 0.821) **
Model 3	1	0.478 (0.294, 0.778) **	0.501 (0.320, 0.840) **	0.473 (0.272, 0.822) **

Model 1: adjusting for age, education, income, labor and exercise status. Model 2: Model 1 + dietary carbohydrate, fat, protein, fiber and cholesterol intake were adjusted. Model 3: Model 2 + smoking habits were adjusted. *: $p < 0.05$; **: $p < 0.01$ compared with the 1st quartile.

Table 5. Associations between dietary histidine and metabolic profile, inflammation and oxidative stress in overweight/obese participants.

	Overall		**Men**		**Women**	
Parameters	***r*** *	***p***	***r*** *	***p***	***r*** *	***p***
FBG	−0.179	<0.05	−0.171	<0.05	−0.214	<0.05
Insulin	−0.122	NS	−0.111	NS	−0.098	NS
HOMA-IR	−0.233	<0.05	−0.221	<0.05	−0.237	<0.05
TC	−0.103	NS	−0.089	NS	−0.109	NS
TG	−0.122	NS	−0.106	NS	−0.127	NS
HDL	0.074	NS	0.033	NS	0.098	NS
LDL	0.081	NS	0.089	NS	0.076	NS
2 h-PG	−0.171	<0.05	−0.167	NS	−0.189	<0.05
2 h-Insulin	−0.112	NS	−0.078	NS	−0.131	NS
2 h-TC	−0.081	NS	−0.065	NS	−0.057	NS
2 h-TG	−0.056	NS	−0.034	NS	−0.078	NS
2 h-HDL	0.098	NS	0.035	NS	0.101	NS
2 h-LDL	0.035	NS	0.008	NS	0.041	NS
GSH-Px	0.265	<0.05	0.231	<0.05	0.283	<0.05
SOD	0.167	<0.05	0.149	NS	0.198	<0.05
MDA	−0.202	<0.05	−0.187	<0.05	−0.231	<0.05
TNF-α	−0.271	<0.05	−0.265	<0.05	−0.273	<0.05

Table 5. *Cont.*

	Overall		Men		Women	
Parameters	***r*** *	***p***	***r*** *	***p***	***r*** *	***p***
IL-1β	−0.178	<0.05	−0.172	<0.05	−0.189	<0.05
IL-6	−0.182	<0.05	−0.166	<0.05	−0.198	<0.05
CRP	−0.242	<0.05	−0.178	<0.05	−0.267	<0.05
Adiponectin	0.188	<0.05	0.176	<0.05	0.195	<0.05
Vaspin	−0.217	<0.05	−0.231	<0.05	−0.203	<0.05

Abbreviations: FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment of insulin resistance; TC, total cholesterol; TG, triglyceride; HDL, high destiny lipoprotein; LDL, low destiny lipoprotein; 2 h-PG, 2 h-postprandial glucose; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malonaldehyde; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; CRP, C-reactive protein; NS, no significance. * Adjusting for dietary carbohydrate, fat, protein, fiber and cholesterol intake.

4. Discussion

This is the first Internet-based cross-sectional study to investigate associations between dietary histidine and prevalence of overweight/obesity and abdominal obesity in northern Chinese adults. We firstly reported that higher dietary histidine intake was associated with lower prevalence of overweight/obesity and abdominal obesity and lower BMI, waist circumference and blood pressure in northern Chinese population, which was firstly found using an Internet-based FFQ study. Moreover, we firstly reported that in overweight/obese individuals, higher dietary histidine is associated with lower FBG, HOMA-IR, 2 h-PG, MDA, TNF-α, IL-1β, IL-6, CRP, MDA and vaspin and higher GSH-Px, SOD and adiponectin.

In this study, we firstly found that dietary histidine is inversely associated with SBP and DBP in northern Chinese population, but no direct evidence was found to support our finding. Only one cross-sectional study reported a positive association between histidine and blood pressure in adolescents [25]. However, the relationship between obesity and hypertension is well established and the mechanisms through which obesity directly causes hypertension are still an area that requires more research [26]. The negative association between dietary histidine and blood pressure can be explained by anti-obesity effect of histidine.

Insulin resistance is the central feature of the metabolic syndrome and is considered as a critical link between obesity and many chronic diseases [27]. To effectively control these chronic diseases, the positive prevention of insulin resistance in obese individuals is extremely important. It has been reported that histidine supplementation could improve insulin resistance in obese women [14]. In the present study, we firstly found that dietary histidine is inversely associated with FBG, HOMA-IR and 2 h-PG in obese individuals, but no significant correlation between dietary histidine and fasting/postprandial insulin was found. These results indicated that dietary histidine also might improve insulin resistance in overweight and obese individuals. However, limited sample size may influence this effect and further studies are needed to confirm this association in normal weight individuals.

It has been confirmed that concentrations of pro-inflammatory cytokines such as TNF-α, IL-6 and CRP are elevated in obese subjects [28] and elevated TNF-α, IL-6 are associated with obesity related insulin resistance [29]. CRP is the most important inflammation biomarker in humans; it is elevated in status of systemic inflammation and is also related to insulin resistance and metabolic syndrome [30]. Therefore, the suppression of pro-inflammation cytokines is considered as an effective strategy for reducing the risk of obesity-related diseases. In animal models of diabetes, histidine supplementation could reduce the levels of IL-6, TNF-α and CRP [31]. Our previous studies in a high-fat diet induced female obese rat model also confirmed that histidine supplementation could ameliorate inflammation and oxidative stress of adipose tissue [11]. In an in vitro study, Son et al. [32] reported that histidine inhibited the hydrogen peroxide- (H_2O_2-) and TNF-α-induced IL-8 secretion at the transcriptional level in intestinal epithelial cells. In a previous study, we had found lower serum histidine concentrations in

obese women than non-obese women. In obese women, serum histidine was negatively associated with inflammation and oxidative stress [13]. Histidine supplementation could improve IR, reduce BMI, fat mass and suppress inflammation and oxidative stress in obese women [14]. In the present study, we firstly found that dietary histidine was inversely associated with TNF-α, IL-1β, IL-6 and CRP, which indicated that dietary histidine also might improve inflammation in overweight and obese individuals. However, this correlation in normal weight individuals is unclear.

Adiponectin is a novel adipocytokine that has been suggested to play a role in the development of insulin resistance and atherosclerosis [33]. Circulating adiponectin levels were decreased in parallel with the development of insulin resistance in rhesus monkeys [34], which indicates that reduced plasma adiponectin levels might play a role in the progress of insulin resistance. In the present study, we also found positive correlation between dietary histidine and serum adiponectin in overweight/obese induviduals. Dietary histidine may increase secretion of adiponectin by suppressing inflammation and oxidative stress in white adipose tissue. Further studies are needed to verify this correlation in normal weight individuals.

Vaspin (visceral adipose tissue-derived serpin) was firstly found in an abdominal obesity with T2DM animal model and was shown as a new adipocytokine to influence insulin sensitivity of white adipose tissues in obese rats. Interestingly, the serum level of vaspin shows increasing trend in prediabetic stage, but decreased with the development of diabetes along with a sharp body weight loss [35]. Circulating vaspin is strongly associated with BMI and insulin sensitivity [36]. A meta-analysis also reporting vaspin levels in subjects with obesity and type 2 diabetes mellitus was higher than healthy controls [37]. In the present study, we firstly found that dietary histidine was inversely associated with serum vaspin in overweight/obese participants. However, the mechanism is unknown.

Oxidative stress is considered as one main cause of insulin resistance and many chronic diseases are characterized by excessive oxidative stress [38]. Therefore, the suppression of oxidative stress is an effective strategy for reducing the risk of obesity-related diseases. Previous study found that the concentrations of SOD in the serum and the mRNA expression of copper zinc superoxide dismutase (CuZnSOD) in the white adipose tissue were increased and the concentrations of MDA in the serum were decreased by histidine supplementation in high-fat diet-induced female obese rat model [11]. In the present study, we quantified three oxidative biomarkers, GSH-Px, SOD and MDA, and the results revealed that the concentrations of GSH-Px and SOD in the serum was positively associated with dietary histidine and the concentrations MDA in the serum was inversely associated with dietary histidine, which provides more evidence that histidine is a potential antioxidant in obese individuals.

For the gender and BMI difference of histidine in our study, we found that negative correlations between dietary histidine and BMI and WC were stronger in overweight and obese participants. Moreover, the association between dietary histidine and overweight/obesity and abdominal obesity is much stronger in women. Several potential mechanisms might account for this finding. In this study, lower histidine intake was observed in female participants and overweight/obese individuals, which may lead to lower serum histidine and stronger response to dietary histidine. Moreover, previous studies found that women were more sensitive to dietary histidine and energy intake than men [15,39], but the mechanisms are still unclear and further studies are needed.

The primary limitation of this study is the cross-sectional and retrospective design. We must acknowledge that reverse causality may be exists as cross-sectional studies were insufficient to confirm the causal relationship between dietary factors and occurrence of disease [17]. Long term cohort studies for this issue are needed to verify the casual relationship; Secondly, current study was only performed in a northern Chinese population. Consider of the large population and vast territory of China, we must be rigorous when extrapolating this result to the general population. Moreover, beneficial effects of dietary histidine such as lower fasting blood glucose, insulin resistance, inflammation and oxidative stress may be associated with histidine peptides (carnosine, anserine and balenine), which has been shown in recent studies of carnosine supplementation in animals [40] and more recently

in humans [41]. However, in our study, data of dietary histidine peptides were lacking. In spite of these limitations, this study provides a lot of practical significance, especially in nutritional counseling. As nutrition is important for prevention of obesity and reduction of excess body weight, reasonable dietary intervention should be useful for the prevention of overweight/obesity. For overweight/obese clients, advice of consuming more histidine-rich foods may be useful for weight control.

5. Conclusions

In summary, our study adds evidence supporting the inverse associations between dietary histidine and obesity, possibly through reduction of energy intake and improvements of insulin resistance, inflammation and oxidative stress. To confirm the casual associations, long-term cohort studies of this issue are needed.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/7/420/s1, Table S1: Characteristics of overall and subgroup participants.

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Review

Probiotics and Prebiotics: Present Status and Future Perspectives on Metabolic Disorders

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Abstract: Metabolic disorders, including type 2 diabetes (T2DM) and cardiovascular disease (CVD), present an increasing public health concern and can significantly undermine an individual's quality of life. The relative risk of CVD, the primary cause of death in T2DM patients, is two to four times higher in people with T2DM compared with those who are non-diabetic. The prevalence of metabolic disorders has been associated with dynamic changes in dietary macronutrient intake and lifestyle changes over recent decades. Recently, the scientific community has considered alteration in gut microbiota composition to constitute one of the most probable factors in the development of metabolic disorders. The altered gut microbiota composition is strongly conducive to increased adiposity, β-cell dysfunction, metabolic endotoxemia, systemic inflammation, and oxidative stress. Probiotics and prebiotics can ameliorate T2DM and CVD through improvement of gut microbiota, which in turn leads to insulin-signaling stimulation and cholesterol-lowering effects. We analyze the currently available data to ascertain further potential benefits and limitations of probiotics and prebiotics in the treatment of metabolic disorders, including T2DM, CVD, and other disease (obesity). The current paper explores the relevant contemporary scientific literature to assist in the derivation of a general perspective of this broad area.

Keywords: metabolic disorders; type 2 diabetes (T2DM); cardiovascular diseases (CVD); gut microbiota; probiotics; prebiotics

1. Introduction

Metabolic diseases, such as type 2 diabetes (T2DM) and cardiovascular diseases (CVD), present an important social problem, considering the increasing morbidity rate in both developing and developed countries. Over the last decade, dynamic changes in dietary macronutrient ingestion and lifestyle have rapidly increased the prevalence of metabolic disorders. T2DM patients have a higher risk of CVD, the primary cause of death. Recently, scientists and nutritionists have proposed that metabolic disorders might result from an alteration in gut microbiota composition [1,2]. *Bacteroidetes* and *Firmicutes* are dominant (>90% of the total microbial population) in human intestine and play a significant role in nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation. Diet controls the composition of these bacteria, which are crucial in the development of metabolic disorders [3–7].

The term "probiotic" originates from the Greek word meaning "for life" [8]. In 1989, Fuller defined the term probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" [8]. In 1995, Gibson *et al.* defined prebiotics, on the other hand, as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating

the growth and/or activity of one or a limited number of bacteria in the colon" [9]. A long history of human consumption of probiotics (particularly *lactic acid bacteria* and *bifidobacteria*) and prebiotics exists, either as natural components of food or as fermented foods. In 76 B.C., the Roman historian Plinius recommended the ingestion of fermented milk products to a patient who had gastroenteritis [10]. Probiotics and prebiotics began to blossom in the late 1800s and early 1900s. Subsequently, Metchnikoff noticed health effects stemming from the alteration of the intestinal microbial balance, and he proposed that the consumption of yogurt containing *Lactobacillus* would result in a decrease in toxin-producing bacteria in the gut and an increase in the longevity of the host [11,12]. In 1900, Tissier recommended the addition of *bifidobacteria* to the diet of infants suffering from diarrhea, claiming that *bifidobacteria* superseded the putrefactive bacteria that caused the condition [13,14]. Since then, numerous scientists have noticed that bacteria in the colon produce many different types of compounds that maintain both positive and negative effects on gut physiology, as well as other systemic influences [15–17]. As an example, short-chain fatty acids (SCFAs) are produced by the fermentation of bacteria, when the bacteria in the colon metabolize proteins and complex carbohydrates. These SCFAs may decrease the risk of developing metabolic disorders due to the increasing demand of cholesterol for *de novo* synthesis of bile acids [18]. Probiotics and prebiotics are considered to be alternative supplements against metabolic disorders, as the manner of their action is thought to be based largely on a modulation of the composition and function of the intestinal microbiota. Several studies have shown that probiotics and prebiotics play an important role in the amelioration of T2DM and CVD [19–21]. A number of researchers studied the potential of food-grade bacteria for treating or preventing diabetes. The studies indicated that certain probiotics (*L. lactis, bifidobacteria*) secrete an insulin analog and promote the expected biological effect on target adipocytes both in human and in animal subjects [22,23]. Accumulating evidence suggests that supplementation of probiotics and prebiotics could have preventative and therapeutic effects on CVD due to a reduction in total serum cholesterol, low-density lipoprotein (LDL-cholesterol), and inflammation [20,24]. This highlights a growing recognition of the role of probiotics and prebiotics in modulating the metabolic activities of the human gut microbiota and regulating the immune system, in turn improving the host's health.

We analyze the current knowledge of the molecular mechanisms by which probiotics and prebiotics participate in host functions that affect the prevention and treatment of metabolic disorders, including T2DM, CVD, and obesity. The current review focuses on the important functions of probiotics and prebiotics through relevant contemporary studies to assist in the derivation of a general perspective of this broad area.

2. Gut Microbiota Compositions and Metabolic Disorders

Interactions between the gut microbiota and the host's overall health begin at birth, and the nature of microbial diversity changes throughout the host's life. The interaction of gut epithelial cells with microbes and their metabolites is a key mediator of the cross-talk between the gut epithelium and other cell types [25]. Additionally, this interaction assists in maturation of the intestinal epithelial layer, the enteric nervous system, the intestinal vascular system, and the mucosal innate immune system. Human gut microbiota are strongly involved in diverse metabolic, nutritional, physiological, and immunological processes, and changes in the composition of the gut microbiota directly influence the host's health [1,26]. Although early intestinal microbiota studies focused on only a minority of bacteria species and their functions, recent researchers have discovered more than 1100 bacteria species and were able to analyse their functional properties as related to certain disease states, such as T2DM, CVD, obesity and cancer, because of the development of advanced techniques, such as DNA-based analyses [27]. In particular, changes of gut microbiota composition are strongly associated with increased adiposity, β-cell dysfunction, metabolic endotoxemia, systemic inflammation, and oxidative stress associated with T2DM [28].

Intestinal microbiota can affect host adiposity and regulate fat storage which, in some cases, can contribute to obesity [3,29]. The change in intestinal microbiota and the reduced bacterial diversity were also observed in obese conditions. For example, Ley *et al.* demonstrated a significant

relationship between gut microbiota composition and obesity. This study showed that the number of *Firmicutes* increased while the number of *Bacteroidetes* decreased in obese mice compared to lean mice [30]. Furthermore, other studies revealed that transplantation of microbiota from obese mice into germ-free mice, despite reduced food intake, significantly increased adipose tissues compared to transplantation of microbiota from lean mice [31]. Larsen *et al.* also demonstrated that the proportions of *Bacteroidetes* to *Firmicutes*were significantly and positively associated with reduction of glucose tolerance. They showed that microbiome diversity was not different between T2DM and non-DM patients, but the composition and function were different, including butyrate-producing bacteria and opportunistic pathogens [32]. The change of these bacteria compositions increases susceptibility to infections, immune disorders, inflammation, oxidative stress and insulin resistance, events that are mediated by metabolic endotoxemia, which involves exposure to noxious intestinal products, particularly lipopolysaccharides (LPS) [33]. LPS is a component of the gram-negative bacteria's cell wall. LPS binds to toll-like receptor-4 (TLR4) on endothelial cells, monocytes,and macrophages. The reaction initiates an inflammatory response and oxidative stress, leading to the activation of NF-κB and AP-1. These activations produce pro- inflammatory cytokines, chemokines, adhesion molecules and reactive oxygen species (ROS), which can cause endothelial damage and dysfunction. For example, trimethylamine N-oxide (TMAO) contributesto the development and progression of cardiovascular disease and the early detection of myocardial injury [34]. TMAO, an oxidation product of trimethylamine (TMA), is a relatively common metabolite of choline in animals [35]. Tang *et al.* validated that increased TMAO levels are associated with increased risk of incidence of major adverse cardiovascular events in a large independent clinical cohort (n = 4007). According to the study, people in the highest quartile of circulating TMAO levels had a 2.5-fold increased risk of having a major adverse cardiac event, when compared to those in the lowest quartile [36]. Furthermore, TMAO levels were dose-dependently related to obesity and insulin resistance in animal studies [37]. Although the mechanisms by which circulating TMAO promotes CVD are currently unclear, there is a possible hypothesis of cardiovascular physiology. Expression of scavenger receptors (CD36 and SR-A1) on macrophages and foam cell formation were increased by supplementation of TMAO in normal chow diet mice [38]. Furthermore, supplementation of TMAO reduces reverse cholesterol transport in macrophage, which would be predicted to advance atherosclerosis [39]. Although supplementation of TMAO clearly influences multiple steps of both forward and reverse cholesterol transport, the underlying molecular mechanisms behind these observations remain unclear. Therefore, further study should be performed to elucidate how circulating TMAO levels are sensed to elicit pathological responses and to explain mechanisms by which TMAO promotes CVD.

Numerous studies also support the theory that gut microbiota can influence host immune functions. Gut microbiota cooperate with the host immune system through an extensive array of signalling pathways, which involve many different classes of molecules and extend beyond the immune system. These immune-mediated signalling processes are directly associated with chemical interactions between the microbe and the host.

3. Probiotics

The definition of a probiotic is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" [40]. The initial concept of probiotics originated from the work of Metchnikoff at the beginning of the 20th century. Subsequently, Shaper *et al.* (1963) and later Mann (1974) observed a reduction in serum cholesterol after consumption of copious amounts of milk fermented with wild *Lactobacillus* and/or *Bifidobacterium* [41,42]. Probiotics have been investigated as a potential dietary supplement that can positively contribute to an individual's health [43]. These health benefits are not limited to the intestinal tract, but also include amelioration of systemic metabolic disorders, such as T2DM and CVD.

Since probiotics have been recognized as a key health promoter thought to stem from the modulation of host immune responses [44], earlier studies have mainly focused on the relationship

between probiotics and immune diseases, such as atopic dermatitis and inflammatory bowel disease. Intestinal bacteria, including *Lactobacilli* and *Bifidobacterium*, can cross the intestinal mucous layer and stimulate phagocytic activities in the spleen or in other organs for many days [45]. Proliferative responses of spleen cells to concanavalin A (a T-cell mitogen) and lipopolysaccharide (a B-cell mitogen) were significantly enhanced in mice supplied with *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, or *Bifidobacterium.* Despite administration of these probiotics, the mice did not exhibit any significant increase in interleukin-4 production by spleen cells nor peripheral blood leucocytes. Instead, spleen cells from mice that consumed these probiotics produced significantly higher amounts of interferon-γin response to stimulation with concanavalin A, compared to cells from the control animals [46].

Several studies have demonstrated that patients with T2DM have a significantly lower number of bacteria that produce butyrate when compared to healthy people. Larsen *et al.* showed an association between T2DM and compositional changes in the intestinal microflora. In particular, they demonstrated a considerably lower proportion of phylum *Firmicutes* and *bifidobacteria* in T2DM patients than in non-diabetic individuals [32,47]. Interestingly, several studies have revealed that probiotics and prebiotics might maintain the potential to improve lipid profiles, including the reduction of LDL-cholesterol, serum/plasma total cholesterol, and triglycerides or increment of high-density lipoprotein (HDL-cholesterol) in the context of treating CVD [22,44,48–52]. Previous studies have proven that the administration of certain probiotics can promote short-chain fatty acids (SCFAs) that alter secretion of incretin hormones and attenuate cholesterol synthesis [53].

4. Prebiotics

A prebiotic was first defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [9]. Subsequently, Roberfroid stated that "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health." [9,54]. Gibson *et al.* examined three criteria, namely: (a) resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; (b) fermentation by intestinal microflora; and (c) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being [55]. Currently, the prebiotics that fulfill these three criteria are fructooligosaccharides, galactooligosaccharides, lactulose, and non-digestible carbohydrates. The non-digestible carbohydrates include large polysaccharides (inulin, resistant starches, cellulose, hemicellulose, pectins, and gums), some oligosaccharides that escape digestion, and unabsorbed sugars and alcohols. Most prebiotics, including fructooligosaccharides and inulin, are digested by *bifidobacteria* and stimulate the growth of their colonies. These bacteria influence homeostasis of intestinal cells and inhibit the growth of pathogenic bacteria [56–58].

SCFAs, such as acetic acid, propionic acid, and butyric acid, are the essential end-products of carbohydrate metabolism. Fermentation of carbohydrates represents a major source of energy for epithelial cells in the colon [59]. SCFAs reduce the development of gastrointestinal disorders, cardiovascular diseases, and cancers by inducing apoptosis (programmed cell death) [18,60]. Furthermore, prebiotics could stimulate the immune system, produce Vitamin B, inhibit pathogen growth, and lower blood ammonia. They also appear instrumental in promoting cell differentiation, cell-cycle arrest, and apoptosis of transformed colonocytes by inhibiting the enzyme histone deacetylase and decreasing the transformation of primary to secondary bile acids [9]. Moreover, SCFAs decrease glucagon levels in a dose-dependent manner, improve glucose tolerance, and activate glucagon-like peptide1 (GLP-1), which can stimulate the elevation of insulin production and increase insulin sensitivity [61,62]. Thus, administration of prebiotics probably plays a regulatory role in modulating endogenous metabolism.

5. Effects of Probiotics and Prebiotics on T2DM

Over recent decades, an abundance of evidence has emerged to suggest a close link between T2DM, CVD, and inflammation. Insulin plays an important role in the regulation of glucose homoeostasis

and lipid metabolism. The failure of target organs to respond to the normal action of insulin is termed *insulin resistance*, which in turn often results in compensatory hyperinsulinemia. This hyperinsulinemia leads to an array of metabolic abnormalities thought to constitute the pathophysiologic basis of metabolic syndrome which can lead to CVD and coronaryheart disease [63].

Moreover, an excess accumulation of visceral fat leads to insulin resistance. In addition, this excess causes a chronic low-grade inflammation characterized by increased macrophage infiltration and pro-inflammatory adipokine production. Pro-inflammatory adipokines obstruct the insulin-signaling pathway in peripheral tissues and promote the development of insulin resistance [63,64]. These data indicate that T2DM is associated with a state of chronic low-level inflammation that leads to the development of CVD. The molecular and cellular underpinnings of obesity-induced inflammation and the signaling pathways at the intersection of metabolism and inflammation contribute to T2DM and CVD [51,52,65].

SCFAs maintain important functions in T2DM patients. Interestingly, some studies have found that the number of SCFAs producing bacteria were significantly lower in people with T2DM. These SCFAs not only bind to G-protein coupled receptors (GPCRs), but also cause the exhibition of various biological effects. For example, SCFAs promote secretion of GLP-1, one of the major incretin hormones primarily synthesized by entero-endocrine L-cells. This hormone inhibits glucagon secretion, decreases hepatic gluconeogenesis, improves insulin sensitivity, and enhances central satiety, resulting in weight loss [66]. Furthermore, some evidence indicates that SCFAs may directly prevent low-grade inflammatory response, as bacteria actively translocate from the intestines into the mesenteric adipose tissue (MAT) and the blood. Amar *et al.* proved that certain probiotics (e.g., *Bifidobacterium animalis* subsp. *lactis* 420) could reverse the low-grade inflammatory response by reducing mucosal adherence and bacterial translocation of gram-negative bacteria from the *Enterobacteriaceae.* As a result, probiotics may attenuate adipose tissue inflammation and several features of T2DM [48]. Asemi *et al.* demonstrated the effects of oral supplements of probiotics on metabolic profiles, high sensitivity C-reactive protein (hs-CRP), and oxidative stress in T2DM. In this randomized, placebo-controlled, and parallel designed study, they utilized an oral supplement comprising seven viable and freeze-dried strains: *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Bifidobacterium breve*, *Bifidobacterium longum*, and *Streptococcus thermophilus.* The test subjects ingested the supplement for eight weeks. The results indicated that the consumption of multi-probiotics led to a meaningful reduction in fasting plasma glucose compared to the placebo group [67].

Additionally, probiotics could promote antioxidation in T2DM patients. Erythrocyte superoxide dismutase, glutathione peroxidase activities, and total antioxidants increased in the group supplemented with probiotic yogurt compared to the control group [68]. Administration of *Lactobacillus acidophilus* and *Lactobacillus casei* with dahi (yogurt in the Indian subcontinent) significantly suppressed streptozotocin (STZ)-induced oxidative damage in pancreatic tissues by inhibiting the lipid peroxidation and nitric-oxide formation [69]. Yadav *et al.* also demonstrated that administration of the probiotic dahi in the diet significantly delayed the onset of glucose intolerance, hyperglycemia, hyperinsulinemia, and dyslipidemia, and decreased oxidative stress in high fructose-induced diabetic rates [70].

In contrast, few papers demonstrated that probiotics fail to maintain significant effects on the lipid profiles of T2DM patients. One of these studies concluded that supplementation of probiotics failed to cause significant changes in total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides (TG), TG/LDL, or LDL/HDL ratios, following eight weeks of intervention [71,72]. Additionally, Lewis *et al.* showed that *lactobacillus acidophilus* administered to 80 hypercholesteraemic volunteers for six weeks failed to produce any significant effects of probiotics on serum blood lipid [73]. Although some studies showed no benefits of probiotics on serum lipids, numerous animal or human studies have demonstrated the benefits of probiotics and prebiotics. Hence, further studies are required to improve our knowledge of, and eliminate uncertainties regarding, probioticsand prebiotics (Tables 1 and 2).

Table 1. Characteristics of the included animal studies.

Intervention Type	Name of Pro/Prebiotic Strains	Study Type	Pro/Prebiotic Type and Dose (Per Day)	Duration of Intervention	Outcomes	Parameter without Change	Reference
Probiotics	*Bacillus*, *Lactobacillus*, *Streptococcus*, *Clostridium*, *Saccharomyces*, *Candida*	Rats	Rice bran (10^7 CFU/g) 30 g/kg	4 weeks	Decreased serum total cholesterol Increase Δ6-desaturase activity and serum arachidonic acid		Fukushima *et al.*, 1999 [74]
Probiotics	*B. lactis* Bb-12, *B. longum* Bb-46	Rats	Buffalo milk yoghurt and soy-yoghurt	4 weeks	Decreased total cholesterol and LDL-C Increasedfecal excretions of bile acids		Abd El-Gawad *et al.*, 2005 [75]
Probiotics	*L. plantarum* PH04	Mice	Human isolate (10^7 CFU/day)	14 days	Decreased total cholesterol and TG Increased fecal lactic acid bacteria		Nguyen *et al.*, 2007 [76]
Probiotics	*L. acidophilus*, *L. casei*, *L. lactis biovar diacetylactis*	Rats	Dahi 15% (150g/kg)	8 weeks	Decreased glucose intolerance, hyperglycemia, hyperinsulinemia, dyslipidemia and oxidative stress	HDL-C	Yadav *et al.*, 2007 [70]
Probiotics	*L. acidophilus* NCDC14, *L. casei* NCDC19	Rats	Dahi (73×10^8 CFU/g)	28 days	Inhibition of insulin depletion, lipid peroxidation and nitrite formation		Yadav *et al.*, 2008 [69]
Probiotics	*B. animalis lactis* 420	Mice	(10^9 CFU/day)	6 weeks	Decreased glucose intolerance, tissue inflammation, insulin resistance and secondarily glycaemia		Amar *et al.*, 2011 [48]
Prebiotics	*Inulin*	Rats	5%	4 weeks	Decrease LDL-C, total cholesterol, Liver lipid and TG concentrations Increased HDL-C, and faecal excretions of bile acids		Kim *et al.*, 1998 [77]

Abbreviations: Bifidobacterium (B), lactobacillus (L), streptococcus (S), colony forming units (CFU), tab (tablet), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein (HDL-C), triglycerides (TG).

Table 2. Characteristics of the included human studies.

Intervention Type	Name of Pro/Prebiotic Strains	Study Type	Pro/Prebiotic Type and Dose (Per Day)	Duration of Intervention	Outcomes	Parameter without Change	Reference
Probiotics	*L. acidophilus* L1,	Human	Fermented milk 200 mL/day	4 weeks	Decreased total cholesterol		Anderson *et al.*, 1999 [78]
Probiotics	*B. longum* BL1	Human/Rats	Fermented milk 100 mL/3 ×day	4 weeks	Decreased total cholesterol, LDL-C and TG	HDL-C	Xiao *et al.*, 2003 [79]
Probiotics	*L. acidophilus* LA-1	Human	Freeze-dried Two tablet/day (3×10^3 CFU/tab)	6 weeks		Total cholesterol, HDL-C, LDL-C, TG	Lewis *et al.*, 2005 [73]
Probiotics	*L. fermentum*	Human	Freeze-dried Two tablet/2 × day (2×10^9 CFU/tab)	10 weeks		Total cholesterol, HDL-C, LDL-C, TG liver enzymes	Simons *et al.*, 2006 [80]
Probiotics	*L. casei subsp. casei.*	Human	Yogurt 100 g/day and 200 g/day	6 weeks	Decreased total cholesterol and LDL-C Increased HDL-C		Fabian *et al.*, 2006 [81]
Probiotics	*L. rhamnosus* LC705, *Propionibacterium freudenreichiissp shermaniistrain* JS	Human	Two tablet/day (2×10^{10} CFU/tab)	4 weeks		Total cholesterol, HDL-C, LDL-C, TG	Hatakka *et al.*, 2008 [82]
Probiotics	*L. acidophilus* La5, *B. lactis* Bb12	Human	Yogurt 300 g/day (2×10^6 CFU/g)	6 weeks	Decreased total cholesterol and LDL-C	HDL-C, TG	Ejtahed *et al.*, 2011 [22]
Probiotics	*L. acidophilus* La5, *B. lactis* Bb12	Human	Yogurt containing 300 g/day (2×10^6 CFU/g)	6 weeks	Decreased fasting blood glucose levels and HbA_1c, Increased erythrocyte superoxide dismutase, glutathione peroxidase activities and total antioxidantstatus	Insulin concentration	Ejtahed *et al.*, 2012 [68]
Probiotics	*L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophiles*	Human	Freeze-dried One tablet/day (14×10^9 CFU/tab)	8 weeks	Decreased serum hs-CRP Increased plasma total GSH Prevention of a rise in fasting plasma glucose		Asemi *et al.*, 2013 [67]

Table 2. *Cont.*

Intervention Type	Name of Pro/Prebiotic Strains	Study Type	Pro/Prebiotic Type and Dose (Per Day)	Duration of Intervention	Outcomes	Parameter without Change	Reference
Probiotics Prebiotics	*L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophiles*, *Fructooligosaccharid-e*	Human	One tablet/day 500 mg/tab	8 weeks	Positive effects on systolic blood pressure	Total cholesterol, LDL-C, HDL-C TG, TG/LDL and LDL/HDL ratios	Mahboobi *et al.*, 2014 [71]
Prebiotics	*Inulin*	Human	Rice-based ready-to-eat cereal (18%)	4 weeks	Decreased total cholesterol and TG Increased breath H2 excretion and fecal lactic acid		Brighenti *et al.*, 1995 [83]
Prebiotics	*Inulin*	Human	One pint of vanilla ice cream (20 g/pint)	3 weeks	Decreased total cholesterol and TG		Causey *et al.*, 2004 [84]

Abbreviations: Bifidobacterium (B), lactobacillus (L), streptococcus (S), colony forming units (CFU), tab (tablet), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein (HDL-C), triglycerides (TG).

6. Effect of Probiotics and Prebiotics on CVD

Cardiovascular disease (CVD) affects blood vessels and/or the heart. CVD primarily stems from hypercholesterolemia and dyslipidemia. Particularly, a high level of LDL-cholesterol is most commonly associated with CVD. CVD represents the most prevalent cause of death in T2DM patients. The relative risk of CVD is two to four times higher in T2DM patients than in non-diabetic people. The most common lipid pattern in people with CVD consists of increased triglyceride-rich lipoproteins, high levels of LDL-cholesterol, and low levels of HDL-cholesterol.

Healthy nutrition and lifestyle intervention constitute important parts of managing CVD. Hypercholesterolemia patients may avoid the use of cholesterol-lowering drugs by practicing dietary control or through administration of probiotics and/or prebiotics. Health food supplements, such as probiotics and prebiotics, can modulate gut health and regulate the immune system through gut microbiota. Persuasive studies have shown that well-established probiotics and/or prebiotics possess hypocholesterolaemic effects in humans and animals. Nguyen *et al.* demonstrated that total serum cholesterol and triglycerides were significantly reduced in hypercholesterolaemic mice that ingested *Lactobacillus plantarum* PH04 [76]. Moreover, some studies supportedthatbuffalo milk yogurt and soymilk yogurt containing *Bifidobacterium* Bb-12 or *Bifidobacterium longum* Bb-46 were highly effective in decreasing the concentration of total cholesterol by 50.3%, LDL- cholesterol by 56.3%, and triglycerides by 51.2% compared to the levels of the control group [75,79,81]. Anderson *et al.* completed a similar study, but they utilized a different probiotic called *Lactobacillus acidophilus* L1. They showed that daily consumption of 200 g of yogurt containing *Lactobacillus acidophilus* after each dinner contributed to a significant reduction in serum cholesterol concentration compared to the placebo group [78]. Another study indicated that the combination of bacteria strains more effectively reduced total cholesterol and liver cholesterol compared to individual bacteria strains. The supplied mixed-bacteria and *Lactobacillus acidophilus* groups exhibited a 23%–57% decrease of cholesterol concentrations in the liver compared to the control group. Additionally, cholesterol concentration in the supplied mixed-bacteria group was lower than in single-bacteria supplemented groups [74].

Prebiotics may lead to hypocholesterolemia via two different mechanisms. First, lower cholesterol absorption is caused by enhanced cholesterol excretion via feces. The other mechanism is the production of SCFAs upon selective fermentation by intestinal bacterial microflora [77]. Causey *et al.* concluded that a daily intake of 20 g of inulin (longer-chain prebiotics, containing 9–64 links per saccharide molecule, fermented more slowly) significantly reduced serum triglycerides compared to the control group. They also found that serum LDL-cholesterol decreased and serum HDL-cholesterol increased following the administration of inulin compared to the control group [84]. Another study showed that when normolipidemic individuals consumed 18% of inulin on a daily basis without any other dietary restrictions, total plasma cholesterol and triacylglycerols decreased by 7.9% $\pm$ 5.4% and 21.2% $\pm$ 7.8%, respectively. Glucose tolerance tests demonstrated that inulin significantly enhanced breath H2 excretion (IAUC test 280 $\pm$ 40; placebo 78 $\pm$ 26 ppm $\times$ h), as well as fecal concentration of *Lactobacillus-lactate* [83]. Thus, inulin may possess lipid-lowering potential in normolipidemic people, possibly mediated by mechanisms related to colonic fermentation. The addition of inulin in the diet of rats induced higher excretions of fecal lipids and cholesterol compared to that of rats in the control group. This increased level of excretion is attributed primarily to reduced cholesterol absorption [85]. Other prebiotics, such as oligodextrans, lactose, resistant starches and their derivatives, lactoferrin-derived peptides, and *N*-acetylchitooligosaccharides have also been identified as maintaining hypocholesterolaemic effects in people with T2DM who are at high risk of developing CVD [55].

Although numerous studies have documented the cholesterol-lowering effects of probiotics and/or prebiotics in both *in vitro* and *in vivo* experiments, the effects remain controversial. Hatakka *et al.* refuted the purported hypocholesterolaemic effect of probiotics, and reported that the administration of *Lactobacillus rhamnosus* LC705 failed to influence blood lipid profiles in 38 men with mean cholesterol levels of 6.2 mmol/L after a four-week treatment period [82]. Lewis *et al.* argued that the administration

of *Lactobacillus acidophilus* failed to affect any serum lipid changes [73]. Furthermore, Simonsa *et al.* showed that a supplement of *Lactobacillus fermentum* failed to significantly change plasma total cholesterol, LDL-cholesterol, HDL-cholesterol, or triglycerides [80]. Although many studies suggest that probiotics can favorably alter serum lipids, some human studies examining the benefits of probiotics on serum lipids have shown conflicting results. This may bedue to the possibility that different delivery systems may affect the experiment result. The human studies, which used capsules probiotics, did not show significant changes inserum lipids compared to fermented bacteria product. A study assumed that sufficient time was not available for the freeze-dried probiotic capsule to become metabolically fully activated before being flushed into the colon. They thought that fermented dairy products can be metabolically active when ingested, whereas freeze-dried probiotic capsules cannot because the small intestinal transit is relatively short [73]. Furthermore, during the intervention, the human studies could not control for an individual's life style, including dietary intake, whereas animal studies could, which may be one of the possible reasons for the apparent lack of effect. Therefore, further researches are required to unequivocally establish the potential role ofprobiotics in the management of metabolic disorder (Tables 1 and 2).

7. Others (Obesity)

Obesity causes low-grade inflammation and an altered composition of the gut microbiota. Some studies have attempted to identify correlations between the composition of the microbiota and the occurrence of inflammation and metabolic alterations in individuals with obesity [86–88]. The low-grade systemic inflammation in the obese phenotype is attenuated by peptides produced in the gut. The composition of gut microbiota affects synthesis of these peptides. One such protein is the serum amyloid A3 protein (SAA3). The gut microbiota serve to regulate SAA3 expression in the adipose tissue [89–91]. Expression of this peptide was considerably higher in the adipose tissue and colon of mice colonized with a normal gut microbiota from a healthy wild-type mouse when compared with germ-free mice [87]. Collectively, these findings suggest that the gut microbiota modulate the biological systems that regulate the availability of nutrients, energy storage, fat mass development, and inflammation in the host, each of which is associated with the obese phenotype [92,93]. Significantly, the number of *bifidobacteria* is inversely correlated with fat mass, glucose intolerance, and LPS level [94,95]. Furthermore, inulin-type fructans affect gut ecology and stimulate immune cell activity. They also decrease weight gain and fat mass in obese individuals [96–98].

8. Molecular Mechanisms of Action

Several hypotheses have been presented to explain how the mechanistic actions of probiotics and prebiotics, including the improvement of gut microbiota, the stimulation of insulin signaling, and the lowering of cholesterol, ameliorate the T2DM and CVD condition. Among the molecular mechanisms, the current paper focuses on SCFA receptors and bile-salt hydrolase (BSH) that are associated with regulation of insulin secretion, fat accumulation, and cholesterol levels.

Recently, two orphan GPCRs, GPR41 (known as FFAR3) and GPR43 (known as FFAR2), were found to be receptors for SCFAs, including acetate, propionate, and butyrate. FFAR2 is primarily activated by acetate and propionate, whereas FFAR3 is more often activated by propionate and butyrate [99]. Both receptors are mainly expressed in L cells, which are located along the length of the intestinal epithelium and respond directly to luminal signals [100]. FFAR2 and FFAR3 stimulate the release of GLP-1 and peptide YY (PYY), which improve insulin secretion. The expression levels of GLP-1 and PYY are often reduced in individuals with T2DM. Therefore, enhancement of GLP-1 and PYY secretion from intestinal L cells could result in beneficial effects in people with T2DM.

Several studies have shown that a deficiency of FFAR2 decreases SCFA-induced secretion of GLP-1 both *in vitro* and *in vivo*, and enhances insulin resistance. The injectable GLP-1 mimetics are associated with good blood glucose control and a decreased incidence of hypoglycemia [100–102]. In addition, FFAR2 regulates energy metabolism via promotion ofleptin secretion, adipogenesis, and inhibition of

lipolysis in adipose tissue and adipocytes [103]. Obesity is frequently observed in FFAR2-deficient mice on a normal diet, while overexpressed FFAR2 in adipose tissue mice remain lean, even though the mice are fed a high-fat diet. Isoproterenol-induced lipolysis is inhibited by SCFSs in a dose-dependent manner in mouse 3T3-L1 derived adipocytes [104,105]. Kimura *et al.* concluded that FFAR2 activation by SCFAs suppressed adipose-specific insulin signaling in white adipose tissues, and thus led to the inhibition of fat accumulation [105].

Similarly, Samuel *et al.* demonstrated that germ-free mice with or without FFAR3 were colonized by specific microbes. The results showed that PYY levels were decreased in FFAR3-deficient mice, indicating that the secretion of PYY from the intestine was regulated by SCFA-induced FFAR3 [106,107]. Moreover, FFAR3 is abundantly expressed in sympathetic ganglia. Inoue *et al.* showed that SCFA-induced FFAR3 activation resulted in increased heart rate and energy expenditure through sympathetic activation. Notably, the effects were not observed in FFAR3-deficient mice. FFAR3 also directly promotes noradrenalin release from sympathetic neurons [108,109]. In contrast, FFAR3 suppresses energy expenditure and produces β-hydroxybutyrate in the liver during starvation. Thus, sympathetic activity is regulated by SCFA-induced FFAR3, thereby maintaining energy balance.

Additional research has indicated that SCFAs are involved in the regulation of hepatic cholesterol synthesis [110,111], as demonstrated via *in vitro* experiments of the liver of germ-free mice. The liver metabolism of germ-free and colonized mice differs considerably, possibly due to the increased influx of SCFAs into the liver of colonized mice [112]. The increased levels of stored triglycerides in the liver and the increased production of the triglyceride transporters were observed in colonized mice. Increased triglyceride synthesis in the liver of colonized mice was associated with reduced expression of fasting-induced adipose factors, or angiopoietin-like 4 (ANGPTL4), in the small intestine. ANGPTL4 inhibits circulating lipoprotein lipase (LPL), which regulates the cellular uptake of triglycerides in adipocytes [113,114]. ANGPTL4 is also a downstream target gene of peroxisome proliferator activated receptors (PPARs), the agonists of which are widely utilized for the treatment of T2DM and CVD [115,116]. PPAR-α mainly plays an important role in hepatic fatty acid oxidation, whereas PPAR-γ constitutes the master regulator of adipogenesis [117]. Moreover, research has indicated that overexpression of ANGPTL4 in the liver leads to decreased activation of LPL and increased plasma triglyceride levels [118]. Interestingly, ANGPTL4 is susceptible to regulation by the gut microbiota [119]. Germ-free ANGPTL4-deficient mice gained considerably more fat mass and body weight compared to colonized mice during high-fat feeding, indicating that ANGPTL4 directly mediates microbial regulation of adiposity in mice [26,120]. Thus, ingestion of SCFAs-producing probiotics could increase influx of SCFAs into the liver, leading to regulation of ANGPTL4 (Figure 1).

SCFA-producing bacteria primarily produce acetate, butyrate, and propionate, which leads to increased FFAR2 and FFAR3 activation. These enhancements of FFAR2 and FFAR3 not only promote noradrenalin release, but also increase heart rate and energy expenditure for energy homeostasis. SCFAs are involved in increased leptin secretion, adipogenesis, and the inhibition of lipolysis in adipose tissues. In the intestine, SCFAs enhance the secretion of PPY and GLP-1. Moreover, an improvement of triglyceride synthesis occurs due to an influx of SCFAs into the liver, which leads to decreased ANGPTL4 activation in the intestines. In addition, SCFA-producing bacteria regulate the suppression of ANGPTL4, an inhibitor of LPL, which promotes increased lipid clearance.

Enzymatic deconjugation of bile acids by bile-salt hydrolase (BSH) has been proposed as an important molecular mechanism in cholesterol-lowering effects. Researchers evaluated BSH's cholesterol-lowering effect utilizing *Lactobacillus plantarum* 80 and *Lactobacillus reuteri*, whereupon it was shown that the enzyme responsible for bile-salt deconjugation in enterohepatic circulation can be detected in probiotics indigenous to the gastrointestinal tract [53,121]. Bile consists of conjugated bile acids, cholesterol, phospholipids, bile pigment, and electrolytes. Synthesized in the liver, bile is stored at high concentrations in the gallbladder between meals. After food intake, it is released into the duodenum. Bile works as a biological detergent that emulsifies and solubilizes lipids for digestion. BSH catalyzes the hydrolysis of glycine or taurine conjugated primary bile acids to create

deconjugated bile acids. The deconjugated bile acids are less soluble and less efficiently reabsorbed than their conjugated counterparts, leading to their elimination in the feces [43,122]. Deconjugation of bile salts can lead to a reduction in serum cholesterol either by increasing the demand for cholesterol for *de novo* synthesis of bile acids to replace those lost in feces or by reducing cholesterol solubility and, thereby, absorption of cholesterol through the intestinal lumen [121,123]. Figure 2 shows the mechanism of enzymatic deconjugation of bile acids by bile-salt hydrolase (BSH).

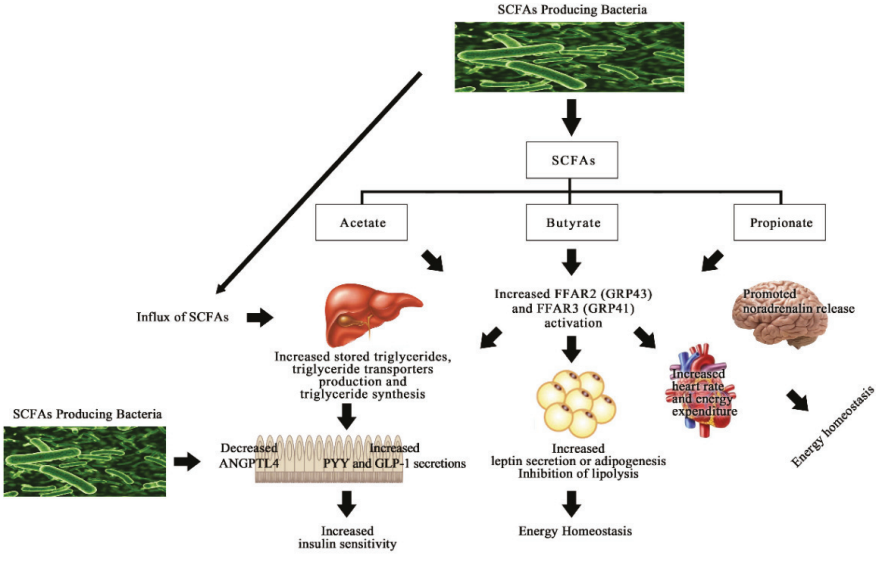

Figure 1. Molecular mechanisms of short-chain fatty acid (SCFA) receptors.

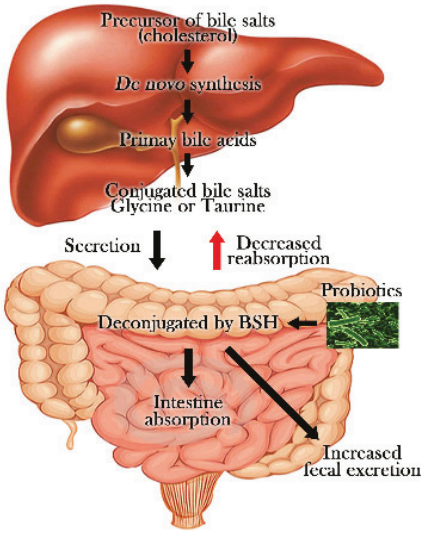

Figure 2. bile-salt hydrolase (BSH) effects on lowering cholesterol by probiotics.

Cholesterol is utilized as the precursor for synthesis of new conjugated bile acids, and the activation of BSH by probiotics catalyzes primary bile acids to create deconjugated bile acids that are less soluble and less efficiently reabsorbed in the intestine and liver. Decongugated bile acids also contribute to the elimination of cholesterol in the feces.

9. Future Prospects

Numerous *in vivo* and/or *in vitro* studies have been conducted utilizing an array of probiotics and/or prebiotics. Key issues in this field are safety and efficacy. Currently, some probiotics (*Lactobacillus, Bifidobacterium*) and prebiotics (inulin, oligofructose) do not require approval from the FDA and are present in our daily dietary intake. Although the safety of probiotics and prebiotics for food application has been confirmed by several legal authorities worldwide, few studies have been conducted regarding incidences of bloating, flatulence, and high osmotic pressure, which can lead to gastrointestinal discomfort [124]. Furthermore, the effects could vary depending on the individual and the type of food containing the prebiotics or probiotics. Probiotics and prebiotics are believed to be safe for oral consumption due to their relatively low capacity to cause adverse effects. However, no standard safety guidelines currently exist for oral administration of probiotics and prebiotics in human cases. Therefore, individual probiotics and prebiotics should be evaluated at specific dosages to ascertain potential adverse reactions.

Although BSH was shown to be beneficial, it may lead to an increase in potentially cytotoxic secondary bile acids in the enterohepatic circulation, which in turn could increase the risk of cholestasis or colorectal cancer [125]. Lithocholic acid (LCA) is a secondary bile acid primarily formed in the intestines by the bacteria. Trauner *et al.* and Beilke *et al.* showed that administration of LCA and its conjugates to animals causes intrahepatic cholestasis. In humans, abnormal bile acid composition, especially an increase in LCA, was found in patients suffering from chronic cholestatic liver disease or cystic fibrosis [126,127]. However, most studies argued mainly for the benefits rather than the adverse effects of BSH from probiotics and/or prebiotics.

The genetic interactions between ingested probiotics and the native intestinal microbes have also constituted a topic of interest. The genetic materials can be exchanged via three mechanisms, including transduction, conjugation, and transformation. The transformation of intestinal microflora by DNA may be enhanced upon the ingestion of bacteria, leading to genetic rearrangements. In addition, the transmission of antibiotic-resistantgenes among beneficial bacteria and harmful pathogens could be associated with a complex microflora colony in the gastrointestinal tract. This transmission can, in turn, lead to the evolution of antibiotic-resistant probiotics and the potential emergence of resistant pathogens [128–131].

10. Conclusions

Metabolic disorders are undoubtedly associated with an increased risk of morbidity and mortality. In our study, we sought to evaluate the effect of probiotics and prebiotics in the context of metabolic disorders. Intestinal microbiota may play an important role in the pathogenesis of T2DM and CVD by influencing body weight, pro-inflammatory activity, and insulin resistance. The scientific community, in general, accepts that the gut microbiota composition and function can be regulated via probiotics and prebiotics. Numerous studies have indicated that probiotics and prebiotics affect T2DM and CVD by changing gut microbiota, regulating insulin signaling, and lowering cholesterol. However, elucidating the interactions between intestinal microbiota and ingested probiotics continues to present a challenge.

Some of the proposed mechanisms and experimental evidence specifically targeting cholesterol-lowering effects remain equivocal. Therefore, more specific and thoroughly designed *in vivo* trials are required to improve our knowledge and eliminate uncertainties. This will, in turn, provide a deeper understanding of the underlying mechanisms and enable us to conduct a more optimal safety assessment prior to the consumption of probiotics and prebiotics by humans. Moreover, no

standard safety guidelines currently exist regarding the oral administration of probiotics and prebiotics in human cases. Therefore, individual probiotics and prebiotics should be carefully evaluated in order to determine potential adverse reactions. Future studies are required to increase our understanding of the complex interplay between intestinal and ingested microbiota.

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nutrients

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Review

The Effect of the Traditional Mediterranean-Style Diet on Metabolic Risk Factors: A Meta-Analysis

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Abstract: The Mediterranean-style diet (MedSD) has gained attention for its positive effects on health outcomes, including metabolic risk factors. However, it is unknown as to which components of MedSD interventions are most beneficial in reducing risk. The objective of this meta-analysis was to obtain effect sizes for metabolic risk factors and explain the variability across the current literature based on study design, sample, and diet characteristics. Six electronic databases were searched from inception until 9 February 2016. Data from 29 studies ($N = 4133$) were included. There were significant effects in favor of the MedSD for waist circumference, triglycerides, blood glucose, systolic blood pressure, and diastolic blood pressure ($d_+ = -0.54$; $d_+ = -0.46$; $d_+ = -0.50$; $d_+ = -0.72$; $d_+ = -0.94$, respectively). The MedSD was significantly beneficial when the intervention was longer in duration, was conducted in Europe, used a behavioral technique, and was conducted using small groups. The traditional MedSD had significant beneficial effects on five of the six metabolic risk factors. Results from this study provide support for population specific dietary guideline for metabolic risk reduction.

Keywords: Mediterranean diet; metabolic syndrome; meta-analysis

1. Introduction

Metabolic syndrome is defined as a group of interrelated risk factors of metabolic origin that appear to directly promote the development of cardiovascular disease (CVD) [1]. The National Cholesterol Education Program's Adult Treatment Panel III report (NCEP ATPIII) [2] identified six components of metabolic syndrome that are related to CVD: (1) abdominal obesity; (2) atherogenic dyslipidemia; (3) elevated blood pressure; (4) insulin resistance; (5) proinflammatory state; and (6) prothrombotic state [2]. According to the ATP III criteria, a diagnosis of metabolic syndrome can be made when three out of five of the following characteristics are present: (1) abdominal obesity characterized by waist circumference (WC) >102 cm for men and >88 cm for women; (2) triglycerides (TG) $\geqslant$150 mg/dL; (3) HDL cholesterol (HDL) <40 mg/dL for men and <50 mg/dL for women; (4) blood pressure $\geqslant$130/$\geqslant$85 mmHg; and (5) fasting glucose (FBG) $\geqslant$110 mg/dL [2]. Metabolic syndrome is a major health concern in the United States. Findings from the Third National Health and Nutrition Examination Survey (NHANES) suggest that according to the NCEP ATP III criteria approximately 34% of adults in the United States have metabolic syndrome [3].

Lifestyle therapies such as diet modification and physical activity are currently recommended as first-line interventions to reduce metabolic risk factors [1]. The Mediterranean-style diet (MedSD) is well-known for its cardio-protective benefits [4] and more recently, has been evaluated for the

prevention and treatment of metabolic syndrome [5]. This dietary pattern emphasizes abundance of plant-based foods, a variety of minimally processed and locally grown foods, and olive oil as the principal source of fat [6]. The MedSD also includes daily consumption of low to moderate amounts of cheese and yogurt (low-fat and non-fat versions may be preferable), twice weekly consumption of fish and poultry, consumption of up to seven eggs per week, fresh fruit as dessert, red meat consumption limited to a few times a month, moderate consumption of wine (1 glass/day for women and 1–2 glasses/day for men) and regular physical activity at a level which promotes healthy weight and well-being [6].

To our knowledge, only one meta-analysis has evaluated literature on the effects of a MedSD on metabolic syndrome [7]. This meta-analysis included 35 clinical trials, two prospective studies, and 13 cross-sectional studies with a total of 534,906 participants and found an overall beneficial effect of the Mediterranean diet on reducing metabolic syndrome and its components in adults [7,8]. Further, the Scientific Report of the 2015 [8] found dietary characteristics similar to that of a MedSD, including higher intake of vegetables, fruits, seafood, legumes, and nuts; moderate intake of alcohol (among adults); lower consumption of red and processed meat, and low intake of sugar-sweetened foods and drinks [8], to have a positive effect on metabolic syndrome risk factors (*i.e.*, blood pressure and lipid profiles). Taken together, the findings from the meta-analysis by Kastorini *et al.* [7] noted above and the 2015 Advisory Committee on the Dietary Guidelines for Americans [8] clearly support the positive effects of the MedSD on metabolic risk factors. However, it is currently unclear which specific characteristics of MedSD-based interventions significantly contribute to the previously observed beneficial effects of a traditional MedSD on metabolic risk factors. We therefore conducted a high quality meta-analysis with specific attention to each criteria of metabolic syndrome, each component of the MedSD, and each methodological characteristic which may help to explain the difference in results between published studies.

2. Methods

2.1. Literature Search

The data sources were obtained following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement [9] guidelines. Original research studies that were published regardless of publication type until 9 February 2016 were included. Language was not restricted. Six computer databases were searched: PubMed, EMBASE (via Scopus), Web of Science, CINAHL, Agricola, and CAB Direct. A comprehensive literature search was conducted with the assistance of the University of Connecticut Health Sciences Librarian (JL) using combinations of Medical Subject Headings and other key words related to the aim of the study. Examples of the key words include: "Mediterranean Diet", "Mediterranean Style Diet", adiposity, "metabolic syndrome", overweight, BMI, "body mass", "waist circumference", obese, obesity, "abdominal fat", and "weight loss". The comprehensive search that was conducted for each database can be found in the supplemental material (S1). In addition to the electronic database search, all studies from Kastorini *et al.* [7] were screened and none of the studies overlap in both meta-analyses due to a difference in inclusion criteria.

2.2. Selection Criteria

Studies had to meet the following criteria to be included: (1) report pre-and post-intervention data on waist circumference (any other metabolic risk factors were additional); and (2) focus on the MedSD as a whole dietary pattern. Studies were excluded if they (1) did not have pre- and post-intervention data on waist circumference; (2) focused on particular components of the Mediterranean diet, such as only olive oil; (3) included exercise in the intervention; (4) included participants <18 years of age; (5) restricted calorie intake; and (6) did not report the information in a way that would allow effect sizes to be calculated using the published information. The relevance of studies was assessed by two

independent researchers (M.G. and J.S.) with a hierarchical approach on the basis of title, abstract, and full manuscript. The original search resulted in 1696 abstracts with relevant key words. After screening and hand-searching articles, 29 articles (39 total comparisons) that used the traditional MedSD were included in analysis. Refer to Figure 1 for the PRISMA figure of included and excluded articles. A list of excluded articles is available in the supplemental material (Table S1).

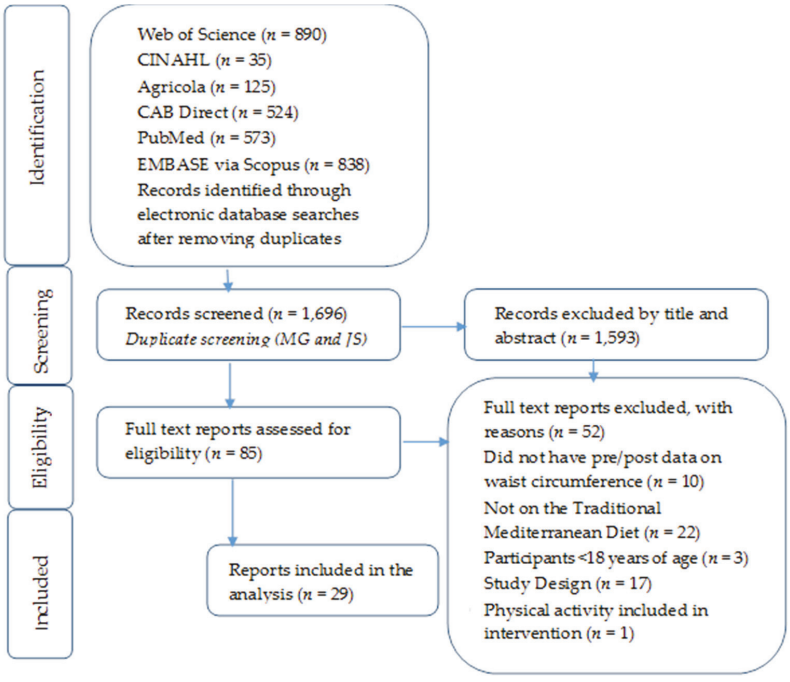

Figure 1. PRISMA Figure outlining the process of study identification, screening, eligibility, and inclusion.

2.3. Data Extraction

A comprehensive and detailed coding form and manual was created by a multidisciplinary team. The coding form includes approximately 330 variables for each study. Various characteristics were extracted from each study: (1) sample characteristics such as ethnicity, number and proportion of females, location of sample, and recruitment details; (2) intervention characteristics such as length of intervention, diet type, distribution of macronutrients, calorie intake, and participation in dietary counseling; and (3) study design characteristics such as number of interventions, type of control group, experimental conditions, and setting. The coding form was pilot-tested by two independent researchers (M.G. and J.S.) and was reviewed by additional experts (J.B., J.K., A.K., T.B.H.-M) before being finalized. The coding form and manual are available upon request to the corresponding author. All 30 studies were independently reviewed and coded by two researchers (M.G. and J.S.) and disagreements were solved by a third-party expert (T.B.H.-M).

2.4. Risk of Bias

The Cochrane Collaboration's tool for assessing risk of bias was used to assess risk of bias within individual studies [10]. In accordance with these guidelines, we report descriptions of internal and

external validity summary ratings categorically, converting these to numerical scores as necessary for the purpose of meta-analytic moderator analysis.

Methodological quality (MQ) rankings have been identified as an under-analyzed element of the data reported in meta-analyses [11]. In this meta-analysis, MQ ratings based on the Cochrane risk of bias scale were entered as one or more possible moderators into the mixed-effects meta-regression models.

2.5. Statistical Analysis

Inter-rater reliability was calculated for all continuous and categorical variables. The kappa (κ) coefficient was used to calculate categorical agreement [12] (kappa = 0.94, 96.9% agreement) and Pearson's correlation coefficient was used to calculate continuous agreement [13] ($r = 1$). We tested for asymmetries by using the Begg [14], Egger [15], and trim-and-fill [16] statistical tests as well as the funnel plot [17] graphical technique. Publication bias, descriptive statistics, and reliability tests were calculated using R version 3.1.2 [18] and particularly, "metafor" package [19] for all the meta-analytic analysis.

Effect sizes (ESs) were calculated for each outcome by calculating the standardized mean change [20] for each sample [21], using the standard deviation of the pretest and adjusting by small sample sizes. The data extracted to obtain the individual ESs could be means and standard deviations, F-ANOVA, *t*-test, or mean and standard deviation change. To uphold the assumption of independence, each outcome was analyzed independently when multiple outcomes were reported from the same study. Twenty-two studies report at least three outcomes with the most common outcomes being waist circumference, HDL cholesterol, and triglycerides. Fourteen studies reported all six outcomes of interest. A multivariate approach for multiple subsamples per study was not followed because no more than five comparisons were available per study. Multiple ESs were obtained from the same study when data was reported separately by participant and diet characteristics [22,23]. Only two studies had subsamples based on sex [24,25] and three studies had multiple subsamples for participant characteristics [26–28].

Weighted mean effect size by the inverse of the variance of each study was calculated across all studies under random- and fixed-effects assumptions [29]. To test for heterogeneity, Cochran's Q [30] and I^2 [31] were calculated. To evaluate the sources of heterogeneity of the ESs, moderator analysis using weighted mixed-effects models with maximum likelihood estimation of the random-effects weights was performed testing each variable for study, intervention, and participant characteristics independently. The moving constant technique [32] was used to produce estimates of the ES ($d+$) at meaningful levels of the moderators and their Confidence Intervals (*Cis*) at different levels of interest. This technique was used to demonstrate results at the maximum and minimum values of significant moderators. Two-sided statistical significance was $p < 0.05$. Finally, clinical units of measures were included by transforming arithmetically the standardized ES to its unstandardized version [33].

3. Results

3.1. Description of Included Studies

A description of the included studies can be found in Table 1. Analysis of 29 reports shows that out of 4133 participants, 72% were female with a mean age of 46.93 (SD = 8.30). A majority of the studies were conducted in Europe (55.9%) and published in English (96.9%). Studies varied in design: 33.3% had a non-MedSD comparison group and 58.9% of studies were crossover or pre-/post-test only design. The mean publication year was 2009 (SD = 2.90) with a 12-year range from 2003 to 2015. The mean intervention length was 35.3 (SD = 50.71) weeks with a range from four to 208 weeks. No significant asymmetries were found using any of the statistical tests or the graphical funnel plot.

Table 1. Description of Included Studies.

Study	Country	*N*	% F	Age	Diseases	Recruit-ment	Dietary Assessment	Type of Diet	Duration (Weeks)	Control	Outcome
Aizawa, *et al.* (2009) [26]	Canada	63	51%	53.9	PDM PHTN	Physician referral	Group, unsupervised	MedSD	24	No carotid artery stiffness	Carotid artery stiffness
Bedard, *et al.* (2012) [27]	Canada	67	NR	39	Ob (57%)	NR	Individual, supervised	MedSD	8	Non-Ob	CVDRF
Bekkouche, *et al.* (2014) [34]	Algeria	86	NR	52	MS (67%)	Hospital	Individual, unsupervised	MedSD	12	No MS, healthy	IR, OS, Inflam.
Bos, *et al.* (2010) [35]	Netherlands	60	NR	52.5	Ob (100%)	NR	Individual, unsupervised	MedSD	10	High SFA diet; High MUFA diet	Serum lipids, IS
Connolly, *et al.* (2011) [28]	Great Britain	206	42%	60.4	CVD or CVDRF (100%)	Hospital, physician referral	Individual, unsupervised	MedSD	16	None	CVDRF
Corbalan, *et al.* (2009) [36]	Spain	1406	82%	39	Ob (100%)	Clinic referral	Individual, unsupervised	MedSD	34	None	WT
Esposito, *et al.* (2006) [37]	Italy	65	0%	43.9	MS, ED (100%)	Research database	Individual, unsupervised	MedSD	24	Regular diet	IIEF score
Esposito, *et al.* (2007) [38]	Italy	59	100%	41.9	MS, FSD (100%)	Research Database	Individual, unsupervised	MedSD	24	Regular Diet	FSFI score
Esposito, *et al.* (2004) [39]	Italy	180	45%	43.9	MS (100%)	Clinic	Group, unsupervised	MedSD	104	Regular Diet	Endo func, Vas Infl
Esposito, *et al.* (2009) [40]	Italy	215	51%	52.2	NIDDM (100%)	Clinic	Group, unsupervised	MedSD	208	LF Diet	Glycemic control
Goulet, *et al.* (2003) [41]	Canada	77	100%	47	None, healthy	Newspaper ad.	Individual, unsupervised	MedSD	12	None	Serum lipid, WT
Goulet, *et al.* (2007) [42]	Canada	77	100%	46.7	None, healthy	Newspaper ad.	Individual, unsupervised	MedSD	24	None	WT
Jones, *et al.* (2011) [43]	United States	89	100%	47.5	MS (100%)	NR	Individual, unsupervised	MedSD-MF	12	MD, no MF	MS RF
Kolomvotsou, *et al.* (2013) [44]	Greece	90	48%	50.4	Ob (100%)	Hospital	Individual, unsupervised	MedSD	8	Regular diet	AO intake, plasma AO capacity

Table 1. *Cont.*

Study	Country	*N*	% F	Age	Diseases	Recruit-ment	Dietary Assessment	Type of Diet	Duration (Weeks)	Control	Outcome
Leblanc, *et al.* (2014) [45]	Canada	108	47%	41.4	Ob, MS (100%)	Media advertise-ments	Individual and group, unsupervised and supervised	MedSD	12	None	Dietary intake, Met profile
Leighton, *et al.* (2009) [46]	Chile	145	0%	39	MS (24%)	Maestranza Diesel	Group, supervised	MedSD	52	None	MS RF
Lerman, *et al.* (2010) [47]	United States	24	83%	54.4	MS and high LDL-C (100%)	Previous study by Lerman	NR	MedSD-MF	12	MD, no MF	Plasma lipids
Lindeberg, *et al.* (2007) [48]	Sweden	29	0%	61	IHD, IGT, NIDDM	Hospital	Individual, unsupervised	MedSD	12	Paleolithic Diet	WT, serum glucose
Llaneza, *et al.* (2010) [49]	Spain	116	100%	56.4	IR (100%)	Hospital	Group, unsupervised	MedSD, soy supplement	104	MD, no supp	IR
Papandreou, *et al.* (2012 [50]	Greece	40	NR	41.5	Ob, OSAS (100%)	University Medical School	Group, unsupervised	MedSD	26	Prudent Diet	OSAS
Papandreou, *et al.* (2012) [51]	Greece	21	NR	41.5	Ob, OSAS (100%)	University Medical School	Group, unsupervised	MedSD	26	Prudent Diet	TBARS
Rallidis, *et al.* (2009) [52]	Greece	82	48%	50.4	Ob (100%)	Hospital	Individual, unsupervised	MedSD	8	Regular Diet	Endo func
Richard, *et al.* (2011) [53]	Canada	26	0%	49.4	MS (100%)	NR	Individual, unsupervised	MedSD	35	Western Diet	CVDRF
Rubenfire, *et al.* (2011) [24]	United States	126	68%	51	MS (100%)	Physician referral	Individual, unsupervised	MedSD	12	None	WT, BP, TG, serum glucose
Ryan, *et al.* (2013) [54]	Australia	12	50%	55	NAFLD (100%)	Hospital	Individual, unsupervised	MedSD	6	LF diet	WT, IS
Sanchez-Benito, *et al.* (2012) [55]	Spain	158	87%	48	OverWT (100%)	Pharmacy office	Individual, unsupervised	MedSD	26	None	BMI, BP, cholesterol
Stendall-Hollis, *et al.* (2013) [56]	United States	129	100%	29.7	OverWT (100%)	Magazine, hospital, Craigslist	Individual, unsupervised	MedSD	16	MyPyramid for P & B	WT, Inflam Bio

Table 1. *Cont.*

Study	Country	*N*	% F	Age	Diseases	Recruit-ment	Dietary Assessment	Type of Diet	Duration (Weeks)	Control	Outcome
Timar, *et al.* (2013) [25]	Romania	223	50%	55	NIDDM (100%)	Diabetes Center	Group, unsupervised	MedSD	52	Diabetic Diet	Glycemic control, CVDRF
Van Velden, *et al.* (2007) [57]	South Africa	12	25%	46	MS (100%)	NR	Group, unsupervised	MedSD with red wine	8	MD without red wine	CVDRF

Note. *N*, number of participants at baseline; F, females; NR, not reported; OverWT, Overweight; Ob, Obesity; MedSD, Mediterranean Style Diet; PDM, Pre-diabetes mellitus; PHTN, Pre-hypertension; CVDRF, Cardiovascular Disease risk factors; MS, Metabolic Syndrome; OS, oxidative stress; NIDDM, Non-insulin Dependent Diabetes; IR, insulin resistance; Inflam, Inflammation; SFA, saturated fatty acid; IS, Insulin Sensitivity; ED, Erectile Dysfunction; IIEF, International Index of Erectile Function; FSD, Female Sexual Dysfunction; FSFI, Female Sexual Function Index; Endo Func., endothelial function; Vas Infl, vascular inflammation; MedSD-MF, Low-Glycemic Mediterranean Diet with Medical Food; MS RF, Metabolic Syndrome Risk Factors; AO, antioxidant; WC, waist circumference; IHD, ischaemic heart disease; IGT, impaired glucose tolerance; OSAS, Obstructive Sleep Apnea Syndrome; TBARS, thiobarbituric acid reacting substances; BP, blood pressure; TG, serum triglycerides; Inflam Bio, inflammatory biomarkers; MyPyramid for P&B, USDA MyPyramid Diet for Pregnant and Breastfeeding Women; FVII, activated factor VII; MI, Myocardial Infarction; Met profile, metabolic profile.

Note on Dietary Assessment column:

- *Individual:* A dietitian performed a dietary assessment, providing individualized needs for caloric intake and recommendations, for each participant.
- *Group:* The study provided general dietary recommendations for the participants, such as a range of servings of certain food groups, calories based on sex, as opposed to tailoring diets to individual needs based on weight and height.
- *Supervised:* Participants consumed foods in a supervised setting, where the researchers had control over participant food choices and quantity of food served.
- *Unsupervised:* Participants food consumption was unsupervised by researchers, such as eating at home.

3.2. Effect Sizes

The traditional MedSD was found to have a significant beneficial effect on five out of six outcomes of interest (Table 2, Figures S1–S6). Overall ESs under random-effects assumptions indicate that the traditional MedSD had a significant overall effect on WC, TG, FBG, systolic blood pressure (SBP), and diastolic blood pressure (DBP) ($d_+ = -0.54$, 95% CI −0.77 to −0.31; $d_+ = -0.46$, 95% CI −0.72 to −0.21; $d_+ = -0.50$, 95% CI −0.81 to −0.20; $d_+ = -0.72$, 95% CI −1.03 to −0.42; $d_+ = -0.94$, 95% CI −1.45 to −0.44, respectively), but did not have a significant effect on HDL ($d_+ = 0.19$, 95% CI −0.07 to 0.46). There was large heterogeneity between studies with I^2 ranging from 92.98% to 98.42%.

Table 2. Summary of Results, Overall Effect Sizes and Homogeneity.

Outcome	*k*	d_+ (95% CI)		Homogeneity of *d*'s		
		Fixed-Effects	Random-Effects	*Q*	I^2 (%)	*p*-Value
WC	39	−0.44 (−0.48 to −0.41) *	−0.54 (−0.77 to −0.31) *	390.1	96.39	<0.0001
HDL	27	0.15 (0.09 to 0.21) *	0.19 (−0.07 to 0.46)	294.6	93.95	<0.0001
TG	25	−0.34 (−0.40 to −0.28) *	−0.46 (−0.72 to −0.21) *	231.06	93.74	<0.0001
FBG	23	−0.37 (−0.42 to −0.33) *	−0.50 (−0.81 to −0.20) *	281.18	96.69	<0.0001
SBP	25	−0.74 (−0.78 to −0.70) *	−0.72 (−1.03 to −0.42) *	320.11	97.00	<0.0001
DBP	25	−0.99 (−1.06 to −0.93) *	−0.94 (−1.45 to −0.44) *	2263.05	98.42	<0.0001

Note: d_+, overall effect size; WC, waist circumference; HDL, HDL cholesterol; TG, triglycerides, FBG, fasting blood glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; * indicates a significant effect; *k* represents the number of interventions for each outcome included in the analysis; *Q* represents Cochran's *Q* indicating significance of heterogeneity; I^2 represents the magnitude of heterogeneity; *p*-value represents the significance of heterogeneity.

3.3. Moderator Analysis

All moderation effects are presented in Table 3. In regards to study characteristics, trending associations were found for study region. Studies conducted in Europe showed significant beneficial effects from the traditional MedSD intervention on four of the metabolic risk factors (waist circumference, HDL cholesterol, triglycerides and fasting blood glucose) whereas studies conducted in the United States did not result in significant effect sizes for any of the study characteristics.

Significant associations were found for study design waist circumference, HDL cholesterol, triglycerides, fasting blood glucose and systolic blood pressure. Studies that included a comparison intervention group design (*i.e.*, a different type of diet) had more beneficial significant effect sizes favoring the MedSD compared to those studies using a traditional pre-/post-design or a crossover design.

Studies with a higher *Impact per Publication* (IPP) value showed more significant beneficial effects for waist circumference, HDL cholesterol, triglycerides, and fasting blood glucose with significant positive associations for each.

The length of the intervention (in weeks) significantly explained between 27.89% and 51.13% of the variability between studies for the following outcomes: waist circumference, HDL cholesterol, triglycerides, fasting blood glucose and systolic blood pressure. There was a significant positive association for length of intervention for all six outcomes of interest (Figures S7–S11). The longer the length of the intervention, the more significant the beneficial effect in favor of the traditional MedSD. Additional significant or trending intervention characteristics included the use of a behavioral technique, supervision, and dietary interventions conducted primarily in small groups. The use of a behavioral technique resulted in trending or significant beneficial effects in all of the outcomes of interest compared to the effects when there was no behavioral technique used.

Table 3. Significant Moderator Analysis Results.

Moderator	Outcome	Category	*k*	d_+ (95% CI)	R^2	*p*-Value	Clinical Unit of Measure
Study Characteristics							
Region	WC	Europe	19	−0.49 (−1.23 to 0.24)	2.25%	0.19	−1.23 cm
		US	7	−0.33 (−0.96 to 0.29)	2.90%	0.19	−0.83 cm
	HDL	Europe	10	0.80 (0.04 to 1.57)	19.3%	0.04	0.13 mmol/L
		US	6	−0.10 (−0.71 to 0.50)	19.3%	0.04	−0.02 mmol/L
	TG	Europe	9	−0.74 (−1.46 to −0.03)	4.11%	0.12	−0.35 mmol/L
		US	4	−0.13 (−073 to 0.46)	4.11%	0.12	−0.06 mmol/L
	FBG	Europe	9	−0.74 (−1.76 to 0.27)	9.14%	0.15	−0.06 mmol/L
		US	3	−0.18 (−1.05 to 0.69)	9.14%	0.15	−0.01 mmol/L
	SBP	Europe	10	−0.68 (−1.83 to 0.47)	0.36%	0.25	−3.21 mmol/L
		US	4	−0.47 (−1.44 to 0.50)	0.36%	0.25	−2.22 mmol/L
	DBP	Europe	10	−1.13 (−2.02 to 1.14)	2.88%	0.24	−3.47 mmol/L
		US	4	−0.44 (−1.95 to 0.74)	2.88%	0.24	−1.35 mmol/L
Study Design	WC	MedSD *vs.* Other Diet	13	−1.14 (−1.49 to −0.78)	28.71%	<0.0001	−2.87 cm
		Pre/Post or Crossover	23	−0.27 (−0.52 to −0.02)	28.71%	<0.0001	−0.68 cm
	HDL	MedSD *vs.* Other Diet	9	0.79 (0.45 to 1.15)	45.64%	<0.0001	0.13 mmol/L
		Pre/Post or Crossover	16	−0.16 (−0.42 to 0.09)	45.64%	<0.0001	−0.16 mmol/L
	TG	MedSD *vs.* Other Diet	8	−0.98 (−1.39 to −0.59)	28.04%	0.008	−0.46 mmol/L
		Pre/Post or Crossover	15	−0.21 (−0.49 to 0.07)	28.04%	0.008	−0.10 mmol/L
	FBG	MedSD *vs.* Other Diet	7	−1.13 (−1.59 to −0.66)	30.92%	<0.0001	−0.09 mmol/L
		Pre/Post or Crossover	14	−0.27 (−0.59 to 0.06)	30.92%	<0.0001	−0.02 mmol/L
	SBP	MedSD *vs.* Other Diet	7	−1.37 (−1.86 to −0.87)	32.26%	<0.0001	−6.47 mmHg
		Pre/Post or Crossover	16	−0.53 (−0.84 to −0.22)	32.26%	<0.0001	−2.69 mmHg
	DBP	MedSD *vs.* Other Diet	7	−1.32 (−2.31 to −0.32)	0.00%	0.004	−4.06 mmHg
		Pre/Post or Crossover	16	−0.87 (−1.52 to −0.21)	0.00%	0.004	−2.67 mmHg

Table 3. *Cont.*

Moderator	Outcome	Category	*k*	d_+ (95% CI)	R^2	*p*-Value	Clinical Unit of Measure
Impact per Publication Metric	WC	0 (minimum)	39	−0.18 (−0.42 to 0.06)	50.13%	<0.0001	−0.45 cm
		16.104 (maximum)	39	−1.89 (−2.43 to −1.37)	50.13%	<0.0001	−4.76 cm
	HDL	0 (minimum)	26	−0.03 (−0.31 to 0.26)	29.44%	0.0006	−0.005 mmol/L
		16.104 (maximum)	26	0.95 (0.38 to 1.52)	29.44%	0.0006	0.15 mmol/L
	TG	0 (minimum)	24	−0.23 (−0.53 to 0.08)	22.65%	<0.0001	−0.11 mmol/L
		16.104 (maximum)	24	−1.09 (−1.68 to −0.52)	22.65%	<0.0001	−0.52 mmol/L
	FBG	0 (minimum)	22	−0.13 (−0.45 to 0.19)	41.52%	0.0004	−0.01 mmol/L
		16.104 (maximum)	22	−1.45 (−2.03 to −0.88)	41.52%	0.0004	−0.11 mmol/L
	SBP	0 (minimum)	24	−0.51 (−0.86 to −0.16)	13.10%	0.13	−2.41 mmHg
		16.104 (maximum)	24	−1.16 (−1.84 to −0.49)	13.10%	0.13	−5.48 mmHg
	DBP	0 (minimum)	23	−0.69 (−1.37 to 0.02)	3.54%	0.18	−2.12 mmHg
		16.104 (maximum)	23	−1.77 (−3.06 to −0.49)	3.54%	0.18	−5.44 mmHg
Intervention Characteristics							
Length of intervention (in weeks)	WC	4 weeks (minimum)	39	−0.24 (−0.45 to −0.03)	46.18%	<0.0001	−0.604 cm
		208 weeks (maximum)	39	−2.50 (−3.29 to −1.71)	46.18%	<0.0001	−6.29 cm
	HDL	4 weeks (minimum)	27	−0.09 (−0.32 to 0.14)	48.04%	<0.0001	−0.01 mmol/L
		208 weeks (maximum)	27	1.79 (1.06 to 2.53)	48.04%	<0.0001	0.29 mmol/L
	TG	4 weeks (minimum)	25	−0.19 (−0.46 to 0.07)	32.1%	0.0009	−0.09 mmol/L
		208 weeks (maximum)	25	−1.73 (−2.51 to −0.95)	32.1%	0.0009	−0.83 mmol/L
	FBG	4 weeks (minimum)	23	−0.19 (−0.45 to 0.07)	51.13%	<0.0001	−0.01 mmol/L
		208 weeks (maximum)	23	−2.22 (−3.02 to −1.41)	51.1%	<0.0001	−0.17 mmol/L
	SBP	4 weeks (minimum)	25	−0.45 (−0.77 to −0.14)	27.89%	0.0004	−2.12 mmHg
		208 weeks (maximum)	25	−2.04 (−2.98 to −1.09)	27.89%	0.004	−9.63 mmHg
	DBP	4 weeks (minimum)	25	−0.67 (−1.26 to −0.08)	6.39%	0.10	−2.06 mmHg
		208 weeks (maximum)	25	−2.37 (−4.15 to −0.59)	6.39%	0.10	−7.28 mmHg

Table 3. *Cont.*

Moderator	Outcome	Category	*k*	d_+ (95% CI)	R^2	*p*-Value	Clinical Unit of Measure
Number of Females	WC	0 (minimum)	35	−0.49 (−0.76 to −0.23)	0.00%	0.95	−1.23 cm
		1154 (maximum)	35	−0.54 (−1.91 to 0.83)	0.00%	0.95	−1.36 cm
	HDL	0 (minimum)	25	0.33 (−0.06 to 0.72)	0.00%	0.39	0.06 mmol/L
		1154 (maximum)	25	−3.43 (−11.69 to 4.83)	0.00%	0.39	−0.56 mmol/L
	TG	0 (minimum)	23	−0.45 (−0.87 to −0.03)	0.00%	0.89	−0.22 mmol/L
		1154 (maximum)	23	−1.04 (−10.91 to 8.83)	0.00%	0.91	−0.49 mmol/L
	FBG	0 (minimum)	21	−0.55 (−0.91 to −0.19)	0.00%	0.91	−0.04 mmol/L
		1154 (maximum)	21	−0.46 (−1.94 to 1.01)	0.00%	0.91	−0.04 mmol/L
	SBP	0 (minimum)	23	−0.70 (−1.04 to −0.36)	0.00%	0.79	−3.31 mmHg
		1154 (maximum)	23	−0.91 (−2.36 to 0.53)	0.00%	0.79	−4.29 mmHg
	DBP	0 (minimum)	22	−0.59 (−0.95 to −0.25)	67.92%	<0.0001	−1.81 mmHg
		1154 (maximum)	22	−5.82 (−7.29 to −4.33)	67.92%	<0.0001	−17.89 mmHg
Total sample size	WC	12 (minimum)	39	−0.54 (−0.81 to −0.26)	0.00%	0.97	−1.36 cm
		1406 (maximum)	39	−0.56 (−1.88 to 0.77)	0.00%	0.97	−1.41 cm
	HDL	12 (minimum)	27	−0.18 (−0.63 to 0.27)	13.29%	0.05	−0.03 mmol/L
		1406 (maximum)	27	5.69 (0.10 to 11.29)	13.29%	0.05	0.93 mmol/L
	TG	12 (minimum)	25	−0.20 (−0.66 to 0.26)	4.55%	0.18	−0.09 mmol/L
		1406 (maximum)	25	−4.65 (−10.79 to 1.48)	4.55%	0.18	−2.22 mmol/L
	FBG	12 (minimum)	23	−0.49 (−0.84 to −0.15)	0.00%	0.85	−0.04 mmol/L
		1406 (maximum)	23	−0.64 (−2.05 to −0.78)	0.00%	0.85	−0.05 mmol/L
	SBP	12 (minimum)	25	−0.71 (−1.05 to −0.35)	0.00%	0.79	−3.35 mmHg
		1406 (maximum)	25	−0.93 (−2.38 to 0.53)	0.00%	0.79	−4.39 mmHg
	DBP	12 (minimum)	24	−0.41 (−0.73 to −0.09)	72.14%	<0.0001	−1.26 mmHg
		1406 (maximum)	24	−5.9 (−7.22 to −4.58)	72.14%	<0.0001	−18.14 mmHg

Table 3. *Cont.*

Moderator	Outcome	Category	*k*	d_+ (95% CI)	R^2	*p*-Value	Clinical Unit of Measure
Sample size of intervention group	WC	11 (minimum)	39	−0.54 (−0.79 to −0.28)	0.00%	0.99	−1.36 cm
		1154 (maximum)	39	−0.55 (−1.93 to 0.82)	0.00%	0.99	−1.38 cm
	HDL	11 (minimum)	27	0.11 (−0.33 to 0.54)	0.00%	0.60	0.02 mmol/L
		1154 (maximum)	27	2.24 (−5.41 to 9.89)	0.00%	0.60	0.37 mmol/L
	TG	11 (minimum)	25	−0.34 (−0.76 to 0.07)	0.00%	0.47	−0.16 mmol/L
		1154 (maximum)	25	−3.45 (−11.62 to 4.73)	0.00%	0.47	−1.65 mmol/L
	FBG	11 (minimum)	23	−0.50 (−0.84 to −0.17)	0.00%	0.96	−0.04 mmol/L
		1154 (maximum)	23	−0.54 (−1.96 to 0.88)	0.00%	0.96	−0.04 mmol/L
	SBP	11 (minimum)	25	−0.71 (−1.05 to −0.37)	0.00%	0.78	−3.35 mmHg
		1154 (maximum)	25	−0.93 (−2.39 to 0.53)	0.00%	0.78	−4.39 mmHg
	DBP	11 (minimum)	24	−0.51 (−0.82 to −0.20)	71.80%	<0.0001	−1.57 mmHg
		1154 (maximum)	24	−5.91 (−7.25 to −4.58)	71.80%	<0.0001	−18.17 mmHg
Use of a behavioral technique	WC	No	21	−0.43 (−0.74 to −0.11)	0.00%	<0.0001	−1.08 cm
		Yes	18	−0.66 (−1.00 to −0.33)	0.00%	<0.0001	−1.66 cm
	HDL	No	14	−0.08 (−0.42 to 0.26)	13.88%	0.02	−0.01 mmol/L
		Yes	13	0.48 (0.13 to 0.83)	13.88%	0.02	0.08 mmol/L
	TG	No	14	−0.27 (−0.61 to 0.06)	6.26%	0.0003	−0.13 mmol/L
		Yes	11	−0.70 (−1.08 to −0.33)	6.26%	0.0003	−0.33 mmol/L
	FBG	No	12	−0.29 (−0.71 to 0.12)	4.51%	0.001	−0.02 mmol/L
		Yes	11	−0.72 (−1.14 to −0.29)	4.51%	0.001	−0.06 mmol/L
	SBP	No	13	−0.53 (−0.94 to −0.12)	1.71%	<0.0001	−2.50 mmHg
		Yes	12	−0.94 (−1.37 to −0.51)	1.71%	<0.0001	−4.44 mmHg
Level of intervention or supervision during the study	WC	Primarily one-on-one	14	−0.47 (−0.83 to −0.11)	17.28%	<0.0001	−1.18 cm
		Small groups	9	−1.14 (−1.58 to −0.69)	17.28%	<0.0001	−2.87 cm
	HDL	Primarily one-on-one	8	−0.18 (−0.63 to 0.28)	15.73%	0.03	−0.03 mmol/L
		Small groups	9	0.65 (0.23 to 1.07)	15.73%	0.03	0.11 mmol/L

Table 3. *Cont.*

Moderator	Outcome	Category	*k*	d_+ (95% CI)	R^2	*p*-Value	Clinical Unit of Measure
Level of intervention or supervision during the study	TG	Primarily one-on-one	8	−0.14 (−0.55 to 0.27)	16.04%	<0.0001	−0.07 mmol/L
		Small groups	7	−1.03 (−1.45 to −0.59)	16.04%	<0.0001	−0.49 mmol/L
	FBG	Primarily one-on-one	7	−0.19 (−0.69 to 0.32)	19.26%	0.0002	−0.01 mmol/L
		Small groups	7	−1.04 (−1.54 to −0.55)	19.26%	0.0002	−0.08 mmol/L
	SBP	Primarily one-on-one	9	−0.48 (−0.92 to −0.04)	28.65%	<0.0001	−2.26 mmHg
		Small groups	7	−1.43 (−1.93 to −0.94)	28.65%	<0.0001	−6.75 mmHg
	DBP	Primarily one-on-one	9	−0.37 (−1.19 to 0.46)	2.44%	0.002	−1.13 mmHg
		Small groups	7	−1.54 (−2.48 to −0.60)	2.44%	0.002	−4.73 mmHg

Note: WC, waist circumference; HDL, HDL cholesterol; TG, triglycerides, FBG, fasting blood glucose; SBP, systolic blood pressure; DBP, diastolic blood pressure; k is the number of interventions included in the analysis for each outcome; R^2 indicates the percentage of heterogeneity that the moderator accounts for; *p*-value represents the significance of the moderation effect; Clinical Unit of Measure was calculated using a predictive model transforming arithmetically the standardized ES to its unstandardized version.

The level of intervention or supervision during the study (*i.e.*, primarily one-on-one or small groups) resulted in significant or trending associations for waist circumference, HDL cholesterol, triglycerides, fasting blood glucose and systolic blood pressure. Interventions consisting of small groups saw significant beneficial effects for all six outcomes, whereas interventions that were primarily one-on-one resulted in only two significant outcomes.

In regards to specific components of the traditional MedSD interventions, specific macronutrient proportions of the diet, assessment of dietary compliance and participant engagement in dietary counseling did not significantly explain the variability between studies. Participant characteristics, in particular the presence or absence of certain disease states, were also analyzed as moderators. Disease states that were included in this analysis were cardiovascular disease, type II diabetes mellitus, metabolic syndrome, and overweight/obesity. These variables were not considered to be significant moderators.

3.4. Risk of Bias

Risk of bias was unclear for random sequence generation, allocation, blinding, incomplete outcome data, selective reporting, and other potential sources of bias (Figure S12). Moderator analysis was not significant for any of the risk of bias parameters (data not shown). No high or low risk of bias was found for random sequence generation and 3.3% of the articles had low risk of bias for allocation concealment. As for blinding of participants and personnel, 6.7% of the articles had low risk of bias and 13.3% of the articles had high risk of bias. Blinding of outcome assessment had 10% low risk of bias and 10% high risk of bias. Incomplete outcome data in the short-term and long-term both resulted in 6.7% of articles with high risk of bias. No high or low risk of bias was reported for selective reporting. With regard to other bias, 3.3% of articles had low risk of bias whereas 10% had high risk of bias.

4. Discussion

The present meta-analysis of 29 intervention trials found that the traditional MedSD has significant beneficial effects for five out of six of the metabolic risk factors: waist circumference, triglycerides, fasting blood glucose, systolic blood pressure and diastolic blood pressure. The significant

heterogeneity between studies was partly attributed to the location of the studies, the length of the intervention, and the IPP value of the journal where the study was published. Significant beneficial associations were found for studies conducted in Europe, those of longer duration, studies using a behavioral technique, studies with a comparison intervention group, and studies conducted primarily in groups for most of the metabolic risk factors. To our knowledge, this is the first meta-analysis to evaluate the effects of the Mediterranean diet on metabolic syndrome that meets 100% of the AMSTAR criteria [58].

Our findings that a traditional MedSD is beneficial in reducing the risk of CVD-associated metabolic parameters complements and extends previous work in this area. Several recent systematic reviews and meta-analyses published on the MedSD and CVD risk have reported similar positive effects on HDL cholesterol [59], triglycerides [59], systolic blood pressure [60,61], diastolic blood pressure [60,61], and fasting blood glucose [60]. These studies also found similar significant positive associations in moderator analysis for studies conducted in Mediterranean countries [7,62], duration of study [7], study design [62], and study quality [4,7].

To our knowledge, only one meta-analysis has been published on the effects of the Mediterranean diet on metabolic syndrome [7]. The meta-analysis by Kastorini *et al.* [7] included 35 clinical trials, two prospective studies, and 13 cross-sectional studies with a total of 534,906 participants. Consistent with our current analysis, Kastorini *et al.* found that the MedSD was associated with reductions in waist circumference, triglycerides and fasting glucose levels. The MedSD was also associated with beneficial effects on HDL cholesterol, whereas there were no association for systolic and diastolic blood pressure [7]. However, in the present meta-analysis we did not find a significant effect for HDL cholesterol and found a significant beneficial association for both systolic and diastolic blood pressure. The literature search employed by Kastorini *et al.* [7] differed from the current meta-analysis in that the search was limited to those manuscripts published in English and to three computer databases. Small literature searches of only a few key terms at a time were conducted rather than one comprehensive literature search. Clinical trials with lack of randomization, lack of a control diet group, and interventions without inclusion of all traditional Mediterranean diet components were excluded from their analysis [7]. For the present meta-analysis, a comprehensive literature search was performed using six electronic databases, language was not restricted and studies without comparison groups or with a lack of randomization were not excluded. Thus, differences in search criteria may have contributed to the reported discrepancies in the associations for the MedSD and HDL cholesterol and the MedSD and blood pressure between the present report and meta-analysis by Kastorini *et al.* [7].

Study Limitation and Strengths

Our meta-analysis had several limitations. There is significant heterogeneity between studies that could not be explained by the moderators included in our analyses. The data reported in our sample of studies did not allow us to control for baseline physical activity or different types and duration of on-going exercise, and thus, physical activity could not be included as a moderator. Weight loss was not the objective in any of the included studies, however, we did not control for weight change among participants. Lastly, ecological fallacy is a possibility as we did not have access to the raw data from the included studies and should be cautious interpreting the group results as individual effects. There are also multiple strengths for this meta-analysis. We used a comprehensive literature search in six electronic databases and an inclusive and comprehensive coding form and manual were used for data extraction. We performed moderation analysis on all variables with sufficient data provided in the published material. We excluded interventions that included exercise, which we believed would have precluded us from solely evaluating diet-associated effects. To our knowledge, this is the first meta-analysis to find significant beneficial associations for MedSD interventions that use behavioral techniques and small group interventions and metabolic risk factors. Lastly, we were able to use the moving constant technique and a predictive model to calculate effect sizes for each significant moderator and transform that effect size into clinical units of measure.

5. Conclusions

The results of the present meta-analysis suggest that the traditional MedSD can have risk reduction effects on a number of metabolic parameters. In addition, the MedSD was significantly beneficial for different metabolic risk factors when, in general the intervention was longer in duration, the study was conducted in Europe, the report was published in a journal with higher Impact per Publication value, the study included a comparison intervention, a behavioral technique was used, and the study was conducted using small groups. More high-quality intervention studies conducted in non-European countries that control for physical activity and changes in weight, and include objective measures of compliance are warranted and would allow for further moderator analyses.

Supplementary Materials: Supplementary Materials are available online at http://www.mdpi.com/2072-6643/8/3/168/s1.

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

Abbreviations

The following abbreviations are used in this manuscript:

MedSD	Mediterranean-style Diet
CVD	Cardiovascular Disease
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
MQ	Methodological Quality
ESs	Effect Sizes
WC	Waist circumference
FBG	Fasting blood glucose
TG	Triglycerides
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
IPP	Impact per publication

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