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Volume 1

Dietary Fructose and Glucose: The Multifacetted Aspects of their Metabolism and Implication for Human Human Health

Edited by
Luc Tappy

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Special Issue Editor

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About the Special Issue Editor

Luc Tappy obtained his MD degree at the University of Lausanne in 1981, and was trained in the Department of Internal Medicine and the Service of Endocrinology, Centre Hospitalier Universitaire Vaudois (CHUV) and in the Diabetes section, Temple University Hospital, Philadelphia, PA. In 2002, he was appointed full professor of physiology and associate physician at the Division of Endocrinology and Metabolism at the CHUV. He was an invited professor at the Centre Hospitalier Sart Tilman in Liège, Belgium (1998–2001), and in the Department of Nutrition at the University of California at Berkeley (1995). His research has essentially focussed on the environmental factors involved in the pathogenesis of obesity and type 2 diabetes. He has conducted a number of studies to evaluate the role of dietary sugars in the development of obesity and insulin resistance, and others aimed at assessing and evaluating the role of sport and physical activity in the prevention of fructose-induced metabolic disorders. He has published more than 200 original articles and review papers in international scientific journals.

Preface to "Dietary Fructose and Glucose: The Multifacetted Aspects of Their Metabolism and Implication for Human Health"

Fructose was identified by the French chemist, Augustin-Pierre Dubrunfaut, in 1847, and its stereochemical properties, together with those of its stereoisomers glucose and galactose, were elucidated in the 1990s by the German chemist, Emil Fisher (REF https://www.acs.org/content/acs/en/molecule-of-the-week/archive/f/fructose.html). This monosaccharide is a product of plant photosynthesis, and hence is a precursor of most dietary macronutrients. Fructose is naturally present in many fruits, vegetables, honey and natural syrups, either under its free, monosaccharide form, or as a constituent of sucrose, a disaccharide made of one molecule of glucose linked to one molecule of fructose. As such, it has always been present in the human diet, but its consumption increased tremendously during the 19th and 20th century due to the colonial trade of sugars and developments of industrial food products (REF Sweetness and power).

Over the past 50 years, fructose metabolism and fructose health effects have attracted considerable attention from biomedical researchers. It started with the elucidation of specific metabolic pathways used for fructose metabolism and the identification of inborn errors of fructose metabolism in humans (REF). Due to the fact that the initial steps of fructose metabolism are not dependent on insulin, and that fructose ingestion does not increase glycaemia to any great extent, there was a renewed interest in fructose as a sugar substitute for subjects with diabetes mellitus in the 1980s. Much of the specific effects of fructose on glucose and lipids homeostasis was acquired from small clinical trials performed during this period. At the turn of the millennium, several investigators raised concern that excess fructose intake may be closely associated with the pathogenesis of obesity and of several non-communicable diseases, such as diabetes, cardio-vascular diseases, non-alcoholic fatty liver diseases, or even cancers and neurodegenerative disorders. This has led to a large increase in the number of studies and publications on fructose and dietary sugars. Knowledge in this field has advanced at a quick pace, yet many issues remain controversial and many novel questions have emerged. The reviews and original articles included in this book encompass a broad range of open questions in the field. It is commonly proposed that dietary fructose causes insulin resistance and dyslipidemia, which may in the long term lead to the development of insulin resistance, diabetes mellitus, and contribute to atherogenesis. The mechanisms underlying these effects however remain controversial. Several reviews and original articles address the relationships between fructose intake and human diseases and discuss possible mechanisms. Novel research perspectives, such as the role of uric acid as a mediator of fructose toxicity, the link between dietary fructose and gut microbiota, or novel molecular targets mediating fructose's adverse effects are proposed in this Special Issue (include here all references 1–15).

When consumed in high amounts, a large proportion of ingested fructose is metabolized in the liver and exerts stress on this organ. There is ever growing evidence that fructose may be instrumental in the development and progression of non-alcoholic fatty liver disease. This has particular relevance for public health since this condition is highly prevalent and is closely associated with insulin resistance in the population. Several articles address potential mechanisms underlying fructose's effects on hepatic de novo lipogenesis, fat accumulation, and liver inflammation. One

clinical study asserts that reducing sugar ingestion can decrease intrahepatic fat content in overweight subjects within 12 weeks. One review proposes that plant polyphenols may offer protective effects on fructose-induced NAFLD (include refs of 16–20).

Prospective cohort studies clearly indicate that a high sugar intake is associated with obesity, and support the hypothesis that sugar intake may play a causal role in body fat gain. Body weight gain is clearly secondary to an excess energy intake, but the reason why dietary sugar drives overfeeding remains hypothetical. It has been proposed that sugar fails to elicit normal satiety signals due to fructose-induced leptin resistance in the brain. It has also been hypothesized that fructose fails to stimulate the release of gut satietogenic factors. Neurosensorial effects of sugars, involving stimulation of sweet taste receptors and activation of mesolimbic dopaminergic reward pathways have also been postulated (include here references of 21–25).

It has long been known that childhood obesity is associated, not only with a high risk of obesity, but also with a high risk of diabetes and cardiovascular diseases during adulthood. Over the past two decades, it has even been robustly documented that maternal nutrition during pregnancy (fetal nutrition) and neonatal nutrition may be strong determinants of metabolic health during adulthood. Several reports address the effects of dietary fructose during pregnancy and early neonatal life on glucose homeostasis and cardiometabolic risk factors (Refs section 26–30).

Finally, fructose may have deleterious effects when consumed in excess in sedentary subjects, but may be a convenient energy substrate for some birds which rely on fructose to build up fat stores before migration, and for athletes for example. Furthermore, physical activity may prevent many of the adverse metabolic effects of a high fructose diet (references of 31–36).

The articles in this book provide a nice overview of fructose science. They illustrate recent scientific knowledge which may link fructose intake to the pathogenesis of obesity and non-communicable diseases. However, they also illustrate that many of the present allegations often presented in the lay press as scientific facts, remain mere hypotheses at this stage, and that still much remains to be discovered about this sugar.

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Special Issue Editor

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Review

Relationship between Added Sugars Consumption and Chronic Disease Risk Factors: Current Understanding

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Abstract: Added sugars are a controversial and hotly debated topic. Consumption of added sugars has been implicated in increased risk of a variety of chronic diseases including obesity, cardiovascular disease, diabetes and non-alcoholic fatty liver disease (NAFLD) as well as cognitive decline and even some cancers. Support for these putative associations has been challenged, however, on a variety of fronts. The purpose of the current review is to summarize high impact evidence including systematic reviews, meta-analyses, and randomized controlled trials (RCTs), in an attempt to provide an overview of current evidence related to added sugars and health considerations. This paper is an extension of a symposium held at the Experimental Biology 2015 conference entitled "Sweeteners and Health: Current Understandings, Controversies, Recent Research Findings and Directions for Future Research". We conclude based on high quality evidence from randomized controlled trials (RCT), systematic reviews and meta-analyses of cohort studies that singling out added sugars as unique culprits for metabolically based diseases such as obesity, diabetes and cardiovascular disease appears inconsistent with modern, high quality evidence and is very unlikely to yield health benefits. While it is prudent to consume added sugars in moderation, the reduction of these components of the diet without other reductions of caloric sources seems unlikely to achieve any meaningful benefit.

Keywords: sucrose; high fructose corn syrup; diabetes; cardiovascular disease; obesity

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1. Introduction

An ancient Hindu fable tells of six learned blind men who approach an elephant. All are highly esteemed, but all are blind. The first blind man approaches the elephant and happens to bump up against its broad and sturdy side and declares "the elephant is very like a wall!" The second blind man feels the tusk and cries an elephant is "very much like a spear!" The third happens to grab the elephant's squirming trunk in his hands and boldly declares the elephant is "very like a snake!" The fourth blind man palpates the leg of the elephant and declares "it is clear the elephant is very like a tree!" The fifth blind man who happens to touch the elephant's ear declares "even the blindest man can tell that the elephant is very like a fan". The sixth blind man happens to grasp the swinging tail and declares to his comrades the elephant is "very like a rope!"

What then ensues is a long, passionate argument filled with heated dispute amongst these learned men which gets them nowhere. Although each is partly right, none of them has seen the whole picture (while learned, they are blind, after all!). This fable has been utilized in many different eras and many different cultures to recount arguments in areas as diverse as theology and politics. It illustrates the inaccuracy of seeing only a part of a subject and assuming that it is the whole. It is a cautionary tale

that even learned men can sometimes be misled by their preconceived notions or only seeing a portion of the whole.

In the complex world of nutrition and particularly in the study of how the foods we eat relate to such chronic conditions as obesity, diabetes and cardiovascular disease (CVD), we are somewhat like the six blind men. Each of us sees a part of the complex puzzle and may assure our colleagues that, in fact, we have solved the entire riddle for how nutrition relates to various disease processes.

The scientific and medical communities have gone down the road of speculating cause and effect without conclusive evidence many times. We blamed salt consumption for contributing to hypertension [1], yet recent evidence suggests that this relationship is far more complex [2,3]. We blamed dietary cholesterol for contributing to heart disease and warned a generation of Americans to avoid eating egg yolks, although that advice has subsequently been found to lack scientific justification [4].

The latest bête noire in nutrition is sweeteners, whether they be nutritive sweeteners, in general, and fructose containing sugars, in particular, or non-nutritive sweeteners (NNS). With the issue of sweeteners, the scientific community faces the problem of trying to offer advice without seeing the totality of the picture, much like the blind men approaching the elephant. It is time to pause and try to see the entire elephant.

This article is based on a symposium conducted at the Experimental Biology Meeting in March 2015, entitled "Sweeteners and Health: Current understandings, controversies, recent research findings and directions for future research". It is our hope that by providing a broad approach to high level evidence related to nutritive sweeteners, we can begin to get a clearer picture of the entire "elephant" about sweeteners and health rather than concluding that the health effects are due to a single component.

Added sugars are among the most controversial and hotly debated topics in all of nutrition [5–22]. Consumption of added sugars has been associated with increased risk of obesity [23–25] as well as increased risk factors for cardiovascular disease (CVD) [26], including dyslipidemia [27,28], elevated blood pressure [20,29,30], diabetes [21,31,32], non-alcoholic fatty liver disease [33,34], and even cognitive decline [35] and cancer [36,37]. Data to support these assertions, however, have been challenged consistently. Often these assertions have been based on research trials which provide added sugars in dosages well above those typically found in human consumption (supraphysiological) [12]. Studies comparing pure fructose to pure glucose, neither which is consumed to any appreciable degree in the human diet, have also been extrapolated to human nutrition [38,39]. Although, some trials have compared sucrose to glucose or starch in isocaloric exchange and demonstrated harm with regard to sucrose in insulin/glucose markers and prediabetes/diabetes. Speculation about chronic conditions based on acute data has frequently been employed [40]. Theoretical models, epidemiologic studies which do not establish cause and effect [31,32,41] or data from animal models which can translate poorly to humans particularly in the areas of nutrition, metabolism, and behavior have further clouded the debate [42–45]. Further controversy has arisen from failure by investigators to clearly acknowledge the limitations of their studies, and misinterpretation or overly simplistic interpretations by media or failure to acknowledge the totality of the evidence often for political reasons or recognition.

A vast amount of literature has been generated, particularly over the past decade, exploring potential linkages between added sugars and various health related conditions. The purpose of this review is to survey some of the modern science, particularly from high quality research trials such as randomized controlled trials, systematic reviews and meta-analyses, in an attempt to provide some clarity in this controversial area. Literature reviews in this manuscript were drawn from articles cited in the World Health Organization report commissioned by Te Morenga et al. [46], articles included in meta-analyses and systematic reviews utilized by the Scientific Advisory Committee on Nutrition (SACN) [47], references utilized by the Dietary Guidelines for Americans 2015–2020 [48], the American Heart Association statement on Carbohydrates and Cardiovascular Disease Risk [49] and randomized controlled trials conducted in the research laboratory of the two authors.

2. Levels of Evidence

Any discussion of health consequences related to added sugars and NNSs must take into account levels of evidence. According to guidelines published both in the United Kingdom and by the US Department of Agriculture (as depicted in Figure 1), the evidence that has the least likelihood of bias is systematic reviews and meta-analyses of randomized controlled trials (RCTs) followed by randomized controlled trials [50]. It should be noted, however, that randomized controlled trials are difficult to apply in the area of nutrition because of the complexity of the field and potential for confounding. Cohort studies (see Table 1) and cross-sectional studies are more prone to bias because of confounding factors that cannot be controlled with this study design. Expert opinion is considered prone to bias as are ecological studies [50].

Table 1. Randomized Control Trials Included.

	Type of Analysis	Findings
Lowndes et al. [51]	50th percentile consumption of fructose containing sugars	No increase in body weight over 10 weeks and no increase in triglycerides. No increase in risk factors for diabetes
Lowndes et al. [52]	Comparison between 10 and 20 percent of calories from either HFCS or sucrose in hypocaloric diets	Significant weight loss occurred in all groups
Lowndes et al. [53]	RCT 355 men and women consuming 8%, 18% or 30% of kcals per days either sucrose or HFCS	Average weight gain over 2 pounds over 10 week period. Mostly driven by 30% kcal per day group. No increased risk factors for diabetes. 10% increase in triglycerides confounded by 2 pound weight gain.
Antar et al. [54]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Bantle et al. [55]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Black et al. [56]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Cooper et al. [57]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Groen et al. [58]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Marckmann et al. [59]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Sorensen et al. [60]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption
Stanhope et al. [61]	Randomized Control Trial	Increase in fasting triglycerides from various levels of added sugar consumption

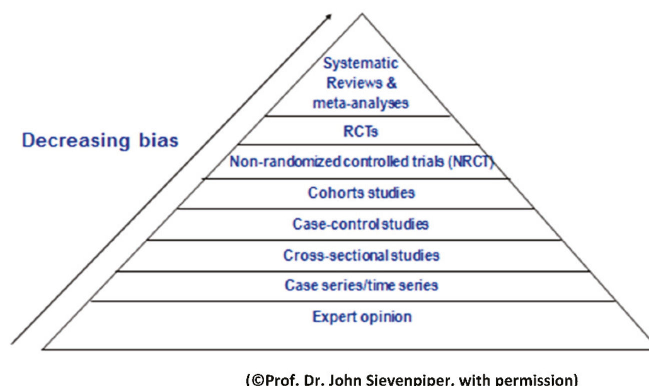

Figure 1. Hierarchy of evidence in evidence based medicine.

3. Controversies Related to Metabolism of Fructose Containing Sugars

Many of the controversies related to fructose related sugars are based on the well-known differences between metabolism of fructose and glucose in the liver [62]. Over 90% of fructose ingested is absorbed through the small intestine and metabolized in the liver on first pass. In contrast, glucose is metabolized by a variety of organs. It is important to note, however, that the pathways are interactive. Numerous studies including isotope studies have shown that roughly 50% of fructose is converted to glucose within the liver. An additional 15%–20% is converted to glycogen, 20%–25% to lactate, and a few percent to carbon dioxide [62,63]. Multiple studies have shown that only 1%–5% of consumed fructose may follow the pathway of de novo lipogenesis and be converted into free fatty acids which are then packaged as triglycerides and either stored in the liver or released in the bloodstream [62,64,65]. Some short-term data with very large doses of pure fructose have suggested that increases in liver fat can be achieved over a short period of time; Faeh et al. gave seven healthy men six days of a high fructose diet comprising an extra 25% of total calories and demonstrated suppression of adipose tissue lipolysis [66].

Schwarz et al. utilizing a diet with 25% pure fructose demonstrated increased fractional hepatic DNL and liver fat [67]. Schwarz et al. studied 25 Latino children and 15 African American children and demonstrated over a ten-day period that replacing high fructose products with vegetables, bread or pasta demonstrated decreased liver fat in this population [68].

In certain animals, de novo lipogenesis can be a major pathway [69]. In humans, it is minimal. Some investigators have misinterpreted the effect of this pathway in humans to contend that fructose consumption can result in increased risk of non-alcoholic fatty liver disease (NAFLD) and insulin resistance [15].

The modern challenge to fructose, in retrospect, came from an opinion piece published in 2004 in the American Journal of Clinical Nutrition by Bray, Nielson and Popkin which asserted that "the increase in consumption of HFCS has a temporal relation to the epidemic of obesity, and the overconsumption of HFCS in calorically sweetened beverages may play a role in the epidemic of obesity" [8]. The authors were careful to point out that this temporal association did not establish cause and effect. It was widely misinterpreted by other scientists and the public at large to suggest that there was something unique about HFCS related to obesity. Subsequent research has shown that HFCS and sucrose have indistinguishable metabolic effects and health consequences in human beings [70–72].

It is also worth noting that sugar consumption has declined significantly in the United States, Britain, Canada, and Australia at a time when obesity rates have continued to rise. This was

first reported in Australia and has become known as the "Australian Paradox" [73]. Furthermore, Mozaffarian et al. reported the impact of increased servings of different food and weight change over a four-year interval by combining Nurses' Health Study (NHSI) (1986–2006), NHSII (1991–2003), and the Health Professionals Follow-up Study (1986–2006) for a combined cohort of a 120,877 people. After multivariable-adjustment for age, Body Mass Index (BMI), sleep, physical activity, alcohol, television watching, smoking and all other dietary factors (French fries, potato chips, processed meat and red meats) all resulted in more weight gain over each four year period than did sugar sweetened beverages (SSB) [74]. These data should be treated with some caution since they come from cohort studies and do not represent a randomized controlled trial. It may be that all of these food products are simply markers for an overall diet that is energy dense and that it is the overall diet pattern, and not any individual component of it, that is associated with weight gain.

4. Effects of Sugars on Body Weight and Body Composition

It has been argued that consumption of sugars may predispose individuals to increase in adiposity, weight gain and ultimately overweight and obesity. A number of randomized controlled trials (RCT) have been performed exploring sugar consumption and weight. These RCTs have been aggregated in four recent meta-analyses, however, these studies employ different inclusion and exclusion criteria and reported different summary endpoint estimates and conclusions [46,75–77] (See Table 2). Sievenpiper et al. [76] and Te Morenga et al. [46] looked at isocaloric exchange of either sugar or fructose with other macronutrients to assess effect of body weight in adults. Neither of these analyses showed significant effect of either sugar or fructose on body weight. With regard to sugars and weight loss Te Morenga et al. reviewed RCTs to examine whether or not the effect of weight and calories from sugars are reduced [46]. These investigators performed meta-analyses on five trials in children and demonstrated no significance in isocaloric trials of children and adults. A meta-analysis by Malik et al. found two of five trials resulted in significant weight loss resulting from a reduction in sugar calories in one model but not another [77]. It should be pointed out that in the trials that were meta-analyzed, subjects consumed not only less calories from sugar, but less total energy. Thus, it is not clear that the weight loss resulted from reduction in calories from sugar.

These four research groups also conducted meta-analyses in studies where an increased amount of sugar calories was given to adults who were consuming ad libitum diets. All four meta-analyses reported significant weight gain in this model although individual studies often did not. Thus, it is not clear whether the change in weight was due to an increase in the total number of calories consumed or some unique property of sugars. Recent meta-analyses by Dolan et al. of interventional studies utilizing the FDA Guidance for Evidence-Based Review both in normal weight [78] and obese individuals [79] did not support a link between obesity and fructose consumption with amounts up to the 90th percentile population consumption for fructose.

The report of the SACN in the UK, which is based on an extensive series of systematic reviews conducted according to clearly stated quality standards, reported that high levels of free sugar consumption were associated with excess energy intake [47]. Thus, weight gain in these studies could not be separated from calorie intake and could not be attributed to any unique property of free sugars. Although it could be argued that free sugar consumption may predispose to excess calorie intake. It has also been reported that fructose containing sugars may predispose individuals to abdominal weight gain [80,81]. If this were true, it would represent a significant increased risk for both diabetes and the metabolic syndrome. Stanhope et al. reported a research trial comparing 25% of calories from fructose to 25% of calories from glucose [81]. Individuals in the fructose arm, over a 10-week period, increased their visceral abdominal fat. However, it should be noted that individuals also gained an average of two pounds over the course of this study. Furthermore, significance in abdominal weight gain occurred only pre-to-post in the fructose arm and this was not compared to the glucose arm. When this more appropriate glucose to fructose comparison was made, the significance disappeared. Maersk et al. [80] conducted a six-month study comparing one liter per day of sugar sweetened

beverage versus comparable amounts of diet beverage, 1% milk, and water. These investigators reported that individuals in the sugar sweetened beverage group increased visceral abdominal fat compared to the other groups. It should be noted, however, that individuals also gained weight in this study which represents a confounding variable.

Table 2. Systematic Reviews and Meta-analyses Included.

	Type of Analysis	Findings
Sievenpiper et al. [76]	Aggregated randomized control trials looking at isocaloric exchange of either sugar or fructose with other macronutrients to assess effects on body weight in adults	No significant effect of either sugar or fructose on body weight
Te Morenga et al. [46]	Aggregated randomized control trials looking at isocaloric exchange of either sugar or fructose with other macronutrients to assess effects on body weight in adults	No significant effect of either sugar or fructose on body weight
Malik et al. [77]	Meta-analysis of 5 trials	2 of 5 trials resulted in significant weight loss from reducing sugar calories in one model but not another
Dolan et al. [78]	Normal weight individuals. Interventional Studies utilizing the FDA guidance for evidence based reviews	No difference with regard to obesity from fructose consumption in normal weight individuals
Dolan et al. [79]	Obese individuals. Interventional Studies utilizing the FDA guidance for evidence based reviews	No difference with regard to obesity from fructose consumption in obese individuals
Cozma et al. [82]	Systematic review and meta-analysis of 18 RCTs	Decrease in risk factors for diabetes such as glycosylated proteins
Malik et al. [24]	Meta-analysis of 8 cohort studies	4 did not find a significant effect of SSB on incidence of diabetes and 5 did not adjust findings for energy intake and body weight
Ha et al. [83]	15 studies involving 355 individuals	Slight decreases in diastolic and mean blood pressure and isocaloric substitution or hypercaloric trials

Three recent RCTs have been conducted employing slightly different strategies have explored aspects of sugar consumption and weight change. In one study, consumption of average amounts of fructose containing sugars for adults (HFCS or sucrose) did not result in increased body weight over a ten-week, free living trial [51]. In another study, mean amounts of these sugars were utilized as part of an overall hypocaloric diet and did not inhibit weight loss [52]. Of note, there were no differences between 10% and 20% of either HFCS or sucrose. In a larger trial involving 355 men and women who consumed either 8%, 18% or 30% of kcals/day of either sucrose or HFCS as part of a mixed nutrient diet, individuals gained an average of slightly over two pounds over a ten-week period. However, most of this was driven by the 30% kcals per day (above the 95% population consumption for fructose) [53]. At the end of the study, individuals consumed an average of more than 200 kcals/day compared to baseline. Thus, this should be viewed as a hypercaloric trial.

Fructose containing sugars led to the expected weight loss (with some exceptions in children) in subtraction trials which suggests that fructose containing sugars do not behave differently from other macronutrients (mainly starch) when comparisons are matched for calories. Another approach to this issue may be obtained from an *ad libitum* trial design where fructose containing sugars were freely replaced with other sources of energy in the diet and no strict control of the amount of sugars in the

background diet occurred. CArbohydrate Ratio Management in European National Diets (CARMEN) trial [84] is the largest and longest trial using such a design. This diet compared ad libitum high complex carbohydrate diet to an ad libitum higher fat control trial in 398 obese individuals studied for over six months. Both ad libitum diets resulted in lost weight. There was no significant different between the ad libitum high sugars diet and the ad libitum high complex carbohydrate diet. There was a non-significant tendency toward greater weight loss in the latter. This trial also showed that under free living conditions it is possible to lose weight following an ad libitum high sugars diet employing a strategy to freely replace energy from high fructose containing sugars with other sources of energy in the diet. It also demonstrates that there is not clear advantage for reducing sugars as compared to fat in the diet [46,75–77]. Given the complexity of weight gain and energy regulation it is unlikely that one component of the diet significantly impacts upon this problem. In fact, the consensus statement from the American Society of Nutrition on energy regulation specifically warns against isolating one component of the diet and blaming it for obesity [85]. Moreover, a large body of literature associates both increased caloric consumption from all sources [86] and decreased physical activity [87] as major components of weight gain. Indeed, the average American consumed 454 more calories in 2010 compared with 1970. Of these additional calories, 93% came from increased consumption of flour and cereal products or fats while only 7% (39 additional calories) came from all sugars combined. The percentage of calories from sugar in the diet in the United States actually declined from 19% to 17% over this period [88]. It should be pointed out, however, that sugars may provide excess energy due to their hedonic properties. In addition, increased sugars intake in some individuals may be a marker for an overall less healthy, energy dense diet.

The recent literature on the impact of added sugars on obesity and weight gain or weight loss remains in dispute. Most of the RCTs suggest that weight gain occurs only in hypercaloric trials and suggests that overall caloric consumption is likely to be a larger contributor to weight gain than any unique property of sugars [74,75].

5. Risk Factors for Diabetes

Considerable confusion exists with regard to the potential impact of added sugars on risk factors for diabetes. A great deal of attention was paid to this issue in the media following two ecological studies which suggested that availability of sugars correlated with increased risk of diabetes [31,32]. These types of ecological studies, however, must be treated with great caution. Ecological studies are considered one of the lowest forms of evidence. Furthermore, these studies have been criticized on a variety of technical grounds. In one ecological study, Goran et al. [32] reported that diabetes prevalence was 20% higher in European Union (EU) countries with higher availability of HFCS compared to countries with low availability. As noted by van Buul et al. however, HFCS consumption data in EU countries reported in this study were, in fact, not consumption data at all but production data [5]. Since HFCS travels freely across EU borders, production data cannot be assumed to be the equivalent of consumption data. In another ecological study, Basu et al. used food supply data from the UNFAO to determine market availability of different food items worldwide and concluded that sugar availability was associated with higher diabetes prevalence. Market availability of food, however, is a highly unreliable indicator of sugar consumption [6].

Prospective cohort studies have not documented a direct relationship between fructose and diabetes [89]. Pooled analysis of these cohorts did reveal that SSBs as a source of free sugar are associated with an increased risk of diabetes only when comparing highest and lowest levels of exposure [22,90]. Pooled analyses of these cohorts, however, for total sugars, total sucrose, and total fructose have not yielded the same relationship [91]. In addition, systematic reviews and meta-analyses of sugar and diabetes risk factors have actually reported a decrease in risk factors such as glycosylated proteins [82]. A large cohort study in Europe also did not show an increase in diabetes risk with added sugars [92].

The question of whether or not sugar is a unique cause of diabetes has not been addressed in any RCT to our knowledge. Most of the data related to the question of a potential relationship between sugar consumption and diabetes comes from RCTs looking at risk factors for diabetes or cohort studies. Prospective cohort studies provide mixed evidence concerning sugar consumption and diabetes. Malik et al. reported meta-analyses of eight cohort studies, four of which did not find a significant effect of SSB with the incidence of diabetes and five did not adjust findings for energy intake and body weight [22]. A study published by the same group did not show a relation between sugar consumption and the risk of diabetes [93]. Other cohort studies have also failed to find significant associations between sugar intake and diabetes [94–96] and one study found a significant negative association [95]. With regard to systematic reviews and meta-analyses, few data are available to support an association between sugar intake and diabetes [94–96]. Cozma et al. reported a systematic review and meta-analysis of 18 feeding studies on fructose and diabetes risk and found no adverse impact on glycemic control including insulin, glucose, glycated blood proteins (including HbA1c) [82]. The SACN report published in 2015 [47] did not show an association between free sugars consumption and risk factors for diabetes.

Most randomized controlled trials of non-diabetic patients substituting sucrose for fructose in a controlled diet did not report adverse effects on multiple risk factors for diabetes [70,78,97–99].

Two recent RCTs have also not demonstrated increased risk factors for diabetes over a 10-week time period. In one study of 123 individuals who consumed average levels of fructose containing sugars (9% of calories from fructose itself or 18% of calories from either sucrose or HFCS) did not yield increases in fasting glucose, insulin, or insulin resistance via the homeostatic model of assessment (HOMA) [100]. Another RCT evaluated 267 individuals who consumed either HFCS or sucrose at dosage ranges between 8% and 30% of calories (25th through 95th percentile of calories) and also did not find any increase in risk factors for diabetes [53].

This literature taken together provides little direct evidence that sugar consumption increases risk factors of diabetes. Moreover, since the relationship between diabetes and obesity is well established and, as already indicated, scant evidence is available relating isocaloric substitution of sugars for other carbohydrates, it appears prudent to focus on other risk factors for diabetes such as obesity rather than singling out sugars. Since diabetes takes 20–30 years to develop short-term RCTs focusing on risk factors for diabetes should be taken with caution recognizing this limitation.

6. Risk Factors for Cardiovascular Disease

The American Heart Association (AHA) has recommended that adult males consume no more than 150 kcals per day and females no more than 100 kcals per day from added sugars [101]. This recommendation implies that higher levels of added sugars may increase the risk of heart disease. In addition, the DGAC 2015 concluded that there was "moderate" evidence in the association between added sugars and heart disease [48]. The SACN report published in 2015 did not find a linkage between sugars consumption and risk factors for heart disease [47]. The evidence in this area, however, is mixed and inconclusive [13]. To our knowledge there are no RCTs assessing a link between added sugars and CVD. Thus, the available data comes either from cohort studies or from RCTs examining risk factors for CVD.

Dietary sugars may have differential effects on blood lipids. A number of studies have demonstrated that diets containing greater than 20% of kcals from simple sugars may result in elevated fasting triglycerides which is a known risk factor for CVD (see Table 3) [32,54–61,99]. The American Heart Association Scientific Statement on triglycerides lists avoiding excess fructose as one mechanism for decreasing the risk of hypertriglyceridemia [102]. Several recent systematic reviews and meta-analyses, however, have reported that in trials where fructose is substituted isocalorically for other carbohydrates it does not result in increased fasting triglycerides or post-prandial triglycerides [103,104].

Table 3. Cohort Studies Included.

	Type of Analysis	Findings
Hodge et al. [94]	Cohort Study	No significant association between sugar intake and diabetes
Meyer et al. [95]	Cohort Study in Older women	Significant negative association between sugar intake and diabetes
Colditz et al. [96]	Cohort Study in women	No association between sugar intake and diabetes
Interact [92]	Cohort Study in European Adults	No increase in diabetes risk with added sugars
Archer et al. [105]	NHANES data analysis	Individuals who consumed 25% or more of calories from added sugars experienced an increase associated risk of cardiovascular disease compared to individuals who consumed less than 10% of calories from added sugars
Yang et al. [106]	NHANES data analysis	CVD risk increased to 1.30 for individuals who consumed 10 to 24.9% of calories and 2.75 for those who consumed 25% or more calories for added sugars compared to individuals who consumed less than 10% of calories from added sugars

Two recent RCTs looked at the relationship between sugar consumption and triglycerides. In one involving 65 individuals where no weight gain occurred, no increase in triglycerides was found [51]. A larger trial involving 355 men and women who consumed between 8% and 30% of kcals per day as either sucrose or HFCS as part of a mixed nutrient diet reported a 10% increase in triglycerides [53]. It should be pointed out, however, that individuals in this trial gained approximately two pounds over the ten-week intervention and were consuming an average of over 200 kcals per day, more by the end of the study compared to baseline. Stanhope et al. followed various doses of HFCS given to young adults over a 16-day period and also reported increases in post-prandial triglycerides [107]. However, the short duration of this study and the fact that pre and post levels were within the low normal range must be taken into consideration when evaluating this finding.

The effects of added sugars on low density lipoprotein (LDL) have been variable [27,59,80,102,108] with some investigators reporting increases while other studies have not demonstrated this finding. It should be noted that a number of the trials where the increases in LDL occurred gave large dosages of added sugars often above the 90th percentile population [109].

A study by Yang et al. published in 2013 analyzed NHANES data from three different time periods (1988–1984, 1999–2004 and 2005–2010) and reported that the relative risk was 1.30 for those who consumed 10%–24.9% of calories from added sugars and 2.75 for those who consumed 25% or more calories from added sugars (approximately 10% of the population) when compared to those who consumed less than 10% of calories from added sugars. It should be noted that the authors also reported that the percentage of daily calories from added sugars was 16.8% in the 1999–2004 cohort and decreased to 14.9% in the 2005–2010 cohort [106]. Several RCTs involving levels of sugar consumption ranging from the 25th to the 95th percentile population consumption have demonstrated no changes in LDL cholesterol following ten weeks in a free living environment compared to baseline when consumed as part of mixed nutrient diet [53]. Thus, the effects of added sugars on lipids in adults remain in dispute.

Research evaluating the effects of added sugars on blood pressure have similarly shown mixed results [29,30,110]. For example, epidemiologic studies such as the Framingham Heart Study have reported an association between consuming one or more SSB per day and increased odds of developing high blood pressure [111]. The meta-analysis by Te Morenga et al. which reported on 12 trials (n = 324) found no significant effects of higher sugar intake on systolic blood pressure overall, although higher sugar intake was associated with significant increase in diastolic pressure of 1.4 mm/hg (95% CI:

0.3, 2.5 mm/hg; p = 0.02) [109]. Many of the trials reported in this systematic review, however, employed amounts of added sugars consumption above the 90th percentile population consumption level. A systematic review and meta-analysis by Ha and colleagues, involving 18 studies (n = 355), showed slight decreases in both diastolic and mean blood pressure when fructose was substituted either isocalorically for other carbohydrates (13 trials) or in hypercaloric trials (2 trials) [83]. Several recent RCTs have not shown increases in blood pressure. In a large study of 355 individuals followed for ten weeks at up to 30% of kcals per day up to the 95% percentile population consumption level of fructose [53], no increases in blood pressure were observed. Further RCTs compared fructose containing sugars to glucose at the 50th percentile population consumption and did not demonstrate increases in mean systolic or diastolic blood pressure [51].

Thus, if there is any association between sugar consumption and increases in blood pressure it would appear to occur at higher levels of sugar consumption (>90th percentile population consumption) and even at that level may not exist.

Taken as a whole, it does not appear that sugar consumption within the normal range of the human diet increases the risk of cardiovascular disease. An exception, however, may occur with diets that contain greater than 20% of kcals from simple sugars in hypercaloric trials which may cause an increase in triglycerides. It should be noted that Archer et al. utilized NHANES data (NHANES 1988–1994, 1999–2004 and 2015) (n = 31,147) compared to the NHANES III Mortality Report (1988–2006) (n = 11,733) and reported that individuals who consumed 25% or more of calories from added sugars (approximately 77% of the population) experienced an increased associated risk of cardiovascular disease [105] compared to those who consumed less than 10% of calories from added sugars. These findings should be treated with caution given the multiple potential confounders inherent to all cohort studies. In particular, NHANES data has recently been challenged because of its use of memory based recall which has been found in multiple studies to be highly inaccurate. These investigators also noted that the percentage of daily calories from added sugars declined from 1999 to 2004 with a decline from 16.8% to 14.9% in 2005–2010 (9% decline).

To put the issue of SSB consumption in perspective, it should be noted that the major risk factors for heart disease are well established such as avoiding cigarette smoking, maintaining a proper weight, avoiding or controlling diabetes and leading a physically active lifestyle. It would appear prudent to focus more attention on these established risk factors than one component of overall approach to nutrition. RCTs of longer duration would be helpful in examining putative links between sugar consumption and risk factors for CVD.

7. Effects of Sugars on the Brain

The effects of sugar on the brain, in general, and on reward pathways, in particular, as well as on downstream portions of the brain has been an area of intense research and controversy. Early studies in this area were done largely on animals [43,112–114], however, recent advances in functional MRI (fMRI) have allowed more studies to be conducted in human beings [115]. Animal studies in this area must be treated with great caution since there are multiple and significant differences between animal brains (in particular, rodents which are the most frequently used model) and human brains [116,117]. Further confusion in this area has come from studies which have utilized a model comparing fructose versus glucose to examine effects on blood flow to the hypothalamus and reward pathways despite the fact that these monosaccharides are rarely consumed by themselves in human nutrition [118,119]. Unfortunately, these trials of two monosaccharides in isolation have led to speculation that fructose and glucose interact differently in the brain thereby leading to potential for overconsumption of calories.

When similar studies have been repeated comparing the normally consumed sugars of sucrose or HFCS on blood flow to the hypothalamus and brain connectively, no differences have been reported between sweetened beverages consumed in the context of a mixed nutrient meal and an unsweetened control [120].

Stice et al. reported a trial of 70 individuals comparing various levels of sugar sweetened milkshakes to various levels of fat in milkshakes and reported that there was more stimulation of reward pathways following the highest level of sugar than fat [40]. These investigators speculated that these acute findings suggested that sugar should be regulated rather than fat with regard to lowering the prevalence of obesity. There are studies, however, which show exactly the opposite [121,122].

Stephan et al. [35] using epidemiologic data suggested that increased consumption of fructose containing sugars could lead to dementia. Studies performed ranging in duration from 10 weeks to 24 weeks and employing average levels of consumption of fructose containing sugars have not found any evidence of cognitive change [123,124].

Unfortunately, some investigators have speculated that sweetness from added sugars may lead to a form of sugar "addiction" [15,125]. Animal data has also been used to buttress this claim [126,127] despite the fact that the translation of animal data to humans in this area is fraught with complexity and speculation. Several recent reviews have provided extensive analyses questioning the fundamental premise of either food or sugar "addiction" [128–130]. Unfortunately, the popular press and the public has embraced the concept of sugar "addiction" which would appear to be a vast exaggeration of what the scientific data show. Clearly, this is an area where much more research is required.

8. Conclusions

There is no question that multiple, important links exist between nutrition and health. The current emphasis on added sugars, however, has created an environment that is "sugar centric" and in our judgment risks exaggerating the effects of these components of the diet with the potential unforeseen side effect of ignoring other important nutritional practices where significant evidence of linkages to health exists.

We have seen the attempt to focus on single nutrients in the diet before attempting to blame a variety of chronic illnesses on overconsumption of these components of the diet [131]. For example, dietary cholesterol was initially blamed as a significant positive factor in coronary artery disease although subsequent research has not supported this linkage. Subsequently, saturated fats were deemed to be a villain although recent evidence now suggests that the food matrix containing the saturated fats may be more important than the saturated fats themselves with regard to risk of CVD [132–134].

The same phenomenon may hold true for isolating components of the diet for supposed health benefits [135]. For example, even though oats have multiple health benefits, the exaggerated health claims caused one pundit to suggest that putting oats in carbonated soft drinks could lead to increase in their sales. There are multiple benefits of consuming protein yet the current fashion of critically accepting high protein diets for a variety of potential health benefits seems overwrought. These are but two of many examples. One has only to look at the popular press to find the current month's super food.

The history of nutrition is littered with attempts to isolate one nutrient, or class of nutrients, to claim a plethora of benefits or risk [131]. These have almost universally resulted in failure and disappointment. In the area of sugar sweetened beverages and various health considerations, the highest quality of evidence from systematic reviews, meta-analyses, and randomized controlled trials does not suggest signals for harm within the normal range of human consumption at least in short-term studies lasting six months or less and in longer-term cohort studies where fructose containing sugars are substituted isocalorically for other carbohydrates. This would suggest that some of the recently articulated restrictive guidelines from prestigious scientific and health organizations may be overly restrictive although longer term studies will be required to provide more certainty on this issue.

We wish to emphasize that we are not recommending excessive consumption of added sugars. It would appear to the authors, however, that a reasonable recommended upper limit of sugar may reside at consuming no more than 20% of calories from added sugars and then only in a hypercaloric

situation. This recommendation rests largely on our view that the evidence suggests a potential signal for elevated triglycerides at consumption levels greater than 20% of calories in hypercaloric trials. We recognize, however, that definitive evidence in this area may be very difficult to generate. Longer term RCTs, particularly, with ad libitum sugar consumption designs may prove helpful. Current ad libitum trials are typically of a short duration.

There are well established risk factors for obesity, diabetes, and cardiovascular disease and considerable overlap amongst these entities when it comes to nutritional practices. For now, we would agree with the assertion in the Dietary Guidelines for American (2010) [136] that overconsumption of calories represents the single greatest health threat to individuals in the United States and elsewhere. This may, in part, be linked to the overall consumption patterns in what has been called the "Western" diet. Certainly, added sugars may be considered as components of this overall diet and, therefore, targets for reduction as are other energy dense components of this nutrition pattern. Singling out added sugars as major or unique culprits for metabolically based diseases such as obesity, diabetes, and cardiovascular disease appears inconsistent with modern high quality evidence and is very unlikely to yield health benefits. The reduction of these components of the diet without other reductions seems very unlikely to achieve any meaningful results. Perhaps in this situation, we should remember a favorite quotation of President John F. Kennedy who quoted Winston Churchill who, in turn, had paraphrased the philosopher George Santayana by saying "Those who fail to learn from history are doomed to repeat it".

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Abbreviations

The following abbreviations are used in this manuscript:

AHA	American Heart Association
CVD	Cardiovascular Disease
fMRI	Functional MRI
HOMA	Homeostatic Model of Assessment
LDL	Low Density Lipoprotein
NAFLD	Non-Alcoholic Fatty Liver Disease
RCTs	Randomized Controlled Trials
SSB	Sugar Sweetened Beverages
NNS	Non-Nutritive Sweeteners

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Individual Diet Modeling Shows How to Balance the Diet of French Adults with or without Excessive Free Sugar Intakes

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Abstract: Dietary changes needed to achieve nutritional adequacy for 33 nutrients were determined for 1719 adults from a representative French national dietary survey. For each individual, an iso-energy nutritionally adequate diet was generated using diet modeling, staying as close as possible to the observed diet. The French food composition table was completed with free sugar (FS) content. Results were analyzed separately for individuals with FS intakes in their observed diets ≤10% or >10% of their energy intake (named below FS-ACCEPTABLE and FS-EXCESS, respectively). The FS-EXCESS group represented 41% of the total population (average energy intake of 14.2% from FS). Compared with FS-ACCEPTABLE individuals, FS-EXCESS individuals had diets of lower nutritional quality and consumed more energy (2192 vs. 2123 kcal/day), particularly during snacking occasions (258 vs. 131 kcal/day) (all p-values < 0.01). In order to meet nutritional targets, for both FS-ACCEPTABLE and FS-EXCESS individuals, the main dietary changes in optimized diets were significant increases in fresh fruits, starchy foods, water, hot beverages and plain yogurts; and significant decreases in mixed dishes/sandwiches, meat/eggs/fish and cheese. For FS-EXCESS individuals only, the optimization process significantly increased vegetables and significantly decreased sugar-sweetened beverages, sweet products and fruit juices. The diets of French adults with excessive intakes of FS are of lower nutritional quality, but can be optimized via specific dietary changes.

Keywords: sugars; linear programming; nutrient recommendations; dietary habits; snacking; France; INCA2

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1. Introduction

In the current context of rising prevalence of non-communicable diseases, sugar intake is increasingly singled out as a public health issue because of its implication in dental caries [1] and weight gain [2], and potentially type 2 diabetes [3,4] and cardiovascular diseases [5–7]. Additionally, higher intakes of added sugars seem to be associated with poorer diet quality and lower micronutrient intakes [8]. Evidence ranges depending on health issues and sugar forms. However, the World Health Organization (WHO) recently focused on the prevention and control of unhealthy weight gain and dental caries, making recommendations for the intake of free sugars in adults and children [9].

For the WHO, the term “sugars” refers to all mono- and disaccharides, and “added sugars” include mono- and disaccharides added to food and beverages by the manufacturer, cook or consumer, and sugars naturally present in honey and syrups, while “free sugars” comprise added sugars plus sugars from fruit juices and concentrates [10].

The WHO recommends reducing the intake of free sugars to less than 10% of energy intake for both adults and children [9]. Today this is the most widely recognized recommendation, though the WHO concurrently makes a “conditional recommendation” of less than 5% of energy intake from free sugars, a threshold adopted by the Scientific Advisory Committee on Nutrition in the UK [11]. More recently, the 2015–2020 Dietary Guidelines for Americans (DGA) recommended limiting energy intakes from added sugars to a maximum of 10% [12]. In Europe, the European Food Safety Agency (EFSA) Panel on “Dietetic Products, Nutrition, and Allergies” declared in 2010 that “there are insufficient data to set an upper limit for (added) sugar intake” [13]. Similarly, in France, no recommendation has been set yet for free sugars.

Worldwide intakes of sugars vary widely by country [13–16] and subject characteristics, such as age [17] and eating patterns, including snacking habits [18]. Additionally, levels of information on sugar intakes (total, added, and free sugars) differ widely among food surveys, with little or no data on free sugars.

In this study, we were able for the first time to characterize the diet of French adults with excessive free sugar intakes, in comparison with those with acceptable free sugar intakes. We then determined the minimum dietary changes needed to achieve adequacy for all nutrients—including 10% maximum energy from free sugars—using diet modeling in individuals with and without excessive intakes of free sugars.

2. Materials and Methods

2.1. Dietary Survey and Population Sample

Data from the French national cross-sectional food consumption survey, named INCA2 (étude Individuelle Nationale des Consommations Alimentaires, 2006–2007) were used in this analysis. This cross-sectional survey, performed on nationally representative samples of children (3–17 years) and adults (18–79 years), using a multi-stage cluster sampling technique, has been described elsewhere [19,20]. To ensure national representativeness, each individual was assigned a weighting factor for unequal sampling probabilities and for differential non-responses. In terms of ethics of human subject participation, this survey was approved by the CNIL, the French authority of data protection (CNIL: “Commission Nationale Informatique et Libertés” No. 2003X727AU) and the CNIS, the French national council for statistical information (CNIS: “Conseil National de l’Information Statistique”). Verbal informed consent was obtained from all participants and formally recorded. The present study focuses on the adult population, aged between 20 and 75 years (n = 2486). Under-reporting individuals (i.e., those who have under-reported their food intake, voluntarily or not), were identified using the Goldberg method, based on the deviation between total energy reported and estimation of energy requirement (based on age, gender, weight, height, physical activity) [21] and excluded from the analysis (26.9% of the total adult sample). Additionally, only respondents who participated in the study for all seven days were retained, which left a final sample of 1726 individuals (Figure S1).

2.2. Demographic, Socio-Economic, Behavioral and Anthropometric Variables

Age, gender, socio-professional status, household type and income, current smoking status, sedentary behavior, frequency of snacking occasions and interest in diet were collected using self-reported and face-to-face questionnaires. Socio-professional status was classified as “active”, “unemployed”, “student”, “retired” or “homemaker”. The household type was described as: “in couple with at least one child”, “in couple with no child”, “single with at least one child” or “single with no child”. Income per consumption unit (ICU) was calculated as self-reported household total net

income divided by the number of consumption units in the household, calculated using the scale from INSEE, the French national institute of statistics and economic studies (INSEE: "Institut National de la Statistique et des Etudes Economiques") [22]. Smoking status was divided into "smoker" and "non-smoker". Frequency of eating between the 3 main meals (breakfast, lunch, and dinner), as declared was divided in five frequencies ("more than four per day", "2–3 times per day", "one time per day", "less than one time per day", or "never"). Three levels of physical activity ("low", "moderate", or "high") were determined according to the short version of the International Physical Activity Questionnaire (IPAQ) [23]. A variable assessing time spent looking at a screen was used as a proxy for sedentariness. This variable was calculated as the sum of the time declared spent in front of the television and computer (including at work), during the week preceding the diet record (minutes (min) per day) [20].

Interest in diet was classified into "a lot", "little", "not really" and "not at all". Trained interviewers measured individual weight and height to calculate body mass index (BMI), divided into four classes (underweight, normal weight, overweight, obesity), according to the WHO definition [24].

2.3. Dietary Assessment

In a seven-day dietary diary, individuals recorded each food and each beverage consumed at home or outside home, split into six moments of consumption: three main meals (breakfast, lunch, and dinner) and three snacking occasions defined as food or beverage consumption between meals (morning, afternoon or evening). During the first face-to-face interview, the diary and a self-administered questionnaire were delivered at home by a trained and certified investigator, who explained to the subjects how to complete them. Just after the survey week, the investigator came back and checked the accuracy of the information reported in both documents [19]. Participants were told to complete the diary during the day in as close as real time as possible, in a pen and paper format. Portion sizes were estimated using a photographic booklet [25] or expressed by weight or household measures (spoon). All foods declared as consumed by the individual during the survey (n = 1314 foods and non-alcoholic beverages, including water) were placed in nine food categories and 30 sub-categories. In addition, to differentiate intrinsic sugar from free sugars, "fruits", "milk" and "yogurts" sub-categories were split into "fresh fruits" and "processed fruits"; "plain milk" and "sweet milk"; "plain yogurts" and "sweet yogurts". The "yogurts" sub-category included yogurts, fermented milks and associated French specialties ("fromage blanc" and "petit-suisses"). Alcoholic beverages were excluded from food analyses because they are not considered as food sources of essential nutrients in dietary recommendations, and therefore could not be optimized.

2.4. Food Composition Database and Free Sugars

The French food composition database [26] was used to estimate the energy and nutrient content of diets. We completed the national food composition table with an additional variable giving the free sugar content of foods. We used the WHO definition [10] which defines free sugars as all monosaccharides and disaccharides added to foods and beverages by the manufacturer, cook or consumer, and sugars naturally present in honey, syrups, fruit juices and fruit juice concentrates. Based on the systematic method to estimate added sugar content [27], the amount of equivalent sugars in all assimilated sugar ingredients was estimated using converting factors (e.g., equivalent sugars accounted for 100% in white sugar and only 80% in honey). Finally, the amount of free sugars for 100 g was estimated using the weight (in the recipe) of assimilated sugar ingredients and their corresponding amounts of sucrose.

In foods from the French food composition table [26], free sugars equal total sugars for 98 foods: honey, 2 syrups and 95 beverages including water. For 627 foods, the amount of free sugars was estimated using average recipes developed by ANSES, the French agency for food, environmental and occupational health and safety (ANSES: "Agence Nationale de SEcurité Sanitaire de l'alimentation, de l'environnement et du travail") and by nutritional expertise. For the remaining 589 foods considered

with no recipe (mainly mono-ingredient foods such as vegetables, non-processed fruits, meats, eggs, fish, etc.), the amount of free sugars was estimated by nutritional expertise, and was nil for 538 of them.

2.5. Diet Quality Indicators

Solid energy density (SED), food variety, mean adequacy ratio (MAR), mean excess ratio (MER), and a diet quality index based on the Probability of Adequate Nutrient intake (PANDiet) were used as indicators of diet quality, and were estimated for each individual observed diet. SED (kcal/100 g) was calculated based on items typically consumed as foods, including soups, but excluded drinking water and items typically consumed as beverages, such as milk, juices and soft drinks [28]. SED was calculated by dividing energy provided by solid foods by their weight. A high SED is associated with low diet quality [29]. Food variety was assessed by the number of different foods declared as consumed by each individual during the 7 days food record [30,31]. As originally proposed, the MAR was used as an indicator of good nutritional quality, and was calculated for each individual observed diet as mean percentages (capped at 100%) of recommended intakes [32] over a week for a list of nutrients. In the present study, it was calculated for 23 key nutrients [33]. The MER, an indicator of poor nutritional quality, was calculated as the mean percentages (minus 100%) of maximum recommended values over a week for sodium, saturated fatty acids and added sugars [33,34]. The updated version of the PANDiet index, integrating free sugars, was also used to estimate the overall nutritional quality of individual diets [35]. It summarizes in a single score the probability of having adequate intakes for 25 positive and negative nutrients. The score ranges from 0 to 100; the higher the score, the better the nutrient adequacy of the diet.

2.6. Diet Modeling

The present modeling approach was based on the previously described Individual Diet models (ID models) [36]. However, to improve its relevance, some changes were made to the original ID models and are described in the Supplementary Materials—Methods. Briefly, the present modeling approach was used to design, for each individual in the dietary survey, a diet at the same energy level which met a set of 33 nutritional recommendations (including 10% maximum energy from free sugars if the intake was greater than 10% or a "no increase" constraint when energy from free sugars was lower than or equal to 10%), while departing the least from the observed diet. To design a diet as similar as possible to the corresponding observed one, the model was parameterized to: (i) preferentially choose repertoire foods (i.e., foods declared as consumed by the individual); (ii) minimize the reduction of the repertoire foods; and (iii) control the introduction of non-repertoire foods (i.e., foods declared as consumed at least once in the survey, but not by this individual). The constraints to be met were a set of nutritional constraints based on dietary reference intakes, a set of acceptability constraints (maximum amounts of foods and food groups) and a set of other constraints, in particular total diet weight and total diet cost (Table S1). "Energy-free" drinks (i.e., drinks containing less than 4 kcal/100 mL) were excluded from the calculation of total diet weight to avoid competition between energy-free drinks and nutrient-dense foods with low energy content.

2.7. Identification of the Most Binding Nutrients

It is possible to identify the constraints the most difficult to fulfill by calculating, for each constraint, a factor named dual value. A null dual value indicates that the constraint is inactive: it has no impact on the optimized solution. In contrast, a non-null dual value means that the constraint is binding or active: it is influencing the result of the optimization process. To identify the most binding constraint, nutritional constraints were ranked in decreasing order according to their percentage of non-null dual values, estimated on the 1719 individuals.

2.8. Statistical Analyses

Of the 1726 adults, the diet optimization was unfeasible for 7 (i.e., no modeled diet able to simultaneously meet all the constraints could be mathematically designed with the list of food variables available for diet modeling). A final sample of 1719 adults was therefore taken for the statistical analysis. Two groups of individuals were defined, depending on the energy contribution from free sugars in their observed diets. Based on the WHO recommendation [9], individuals with a contribution greater than 10% were assigned to the "FS-EXCESS" group (excessive free sugar intakes), and those who had a contribution lower or equal to 10% were assigned to the "FS-ACCEPTABLE" group (acceptable free sugar intakes).

Individual characteristics were described and compared between FS-ACCEPTABLE and FS-EXCESS groups using a chi-squared test for categorical variables and general linear model (GLM) for continuous variables, with and without adjustment for gender and age.

Mean observed nutritional intakes, diet quality indicators, and intakes from food categories and sub-categories (as well as from fresh and processed fruits, plain and sweet milk and plain and sweet yogurts) were described for the whole sample and the two groups. Comparisons of observed food intake, nutritional intake and diet quality indicators between FS-EXCESS and FS-ACCEPTABLE individuals were made using GLM. Observed energy and free sugar intakes from main meals and from snacking occasions were also described and compared between the FS-ACCEPTABLE and FS-EXCESS groups with GLM.

GLM were used to compare the characteristics of observed and optimized diets in the two groups and to compare the variation in grams between optimized and observed diets among FS-EXCESS and FS-ACCEPTABLE individuals. To study changes in sugar balance after diet modeling, the variation in total, free and non-free sugars between observed and optimized diets, from main food categories and sub-categories were calculated and compared using GLM.

Observed energy intake, age and gender were used as a first set of adjustment variables. In a second set of adjustment variables, the current smoking status, BMI, socio-professional status were added to the first set, and, in a third set of adjustment variables, the composition of the family and sitting time were added to the second set. All values were survey-weighted and all analyses accounted for the complex INCA2 sampling frame design [19]. The Operational Research and the STAT packages of SAS version 9.4 (SAS Institute, Cary, NC, USA) were used to run linear programming models and perform statistical analysis, respectively. An alpha level of 1% was used for all statistical tests.

3. Results

3.1. Sample Characteristics

In this representative sample of French adults (n = 1693, weighted value), 41% of individuals (FS-EXCESS group, n = 690) had mean free sugar intakes above the 10% of energy intake level recommended by the WHO (mean intake 14.2% $\pm$ 4.2% of energy intake) and 59% (FS-ACCEPTABLE group, n = 1003) had acceptable intakes, i.e., below 10% of energy intake (mean intake 6.3% $\pm$ 2.5% of energy intake).

Demographic, anthropometric, socio-economic and behavioral characteristics are given in Table 1. Individuals were on average 10 years younger in the FS-EXCESS group than in the FS-ACCEPTABLE group. Individuals in the FS-EXCESS group had a lower BMI (23.6 vs. 25.2 kg/m^2), and the percentage of overweight or obese individuals among them was lower than in the FS-ACCEPTABLE group, even after adjustment for age and gender. In the FS-EXCESS group, the percentages of single individuals and couples with children were higher than in the FS-ACCEPTABLE group, while the percentage of couples without children was lower. In addition, the percentages of professionally active people and students were higher, while the percentage of retirees was lower in the FS-EXCESS group than in the FS-ACCEPTABLE group. There were proportionately more smokers in the FS-EXCESS group; this difference between groups was no longer significant after adjustment for age and gender.

Table 1. Demographic, anthropometric, socio-economic and behavioral characteristics of the total sample, FS-ACCEPTABLE and FS-EXCESS groups.

	ALL	FS-ACCEPTABLE	FS-EXCESS	p [1]	p [2]
Individuals, n	1693	1003	690		
Age, year [3]	47.0 ± 15.02	51.1 ± 14.0	41.1 ± 14.5	<0.001	-
Age, %					
20–34	25.4	15.1	40.3		
35–49	29.8	28.9	31.3		
50–64	29.0	35.0	20.3		
65–75	15.8	21.0	8.2		
Gender, %				0.087	-
Male	47.6	49.4	45.0		
Female	52.4	50.6	55.0		
BMI, kg/m^2 [3,4]	24.5 ± 4.3	25.2 ± 4.3	23.6 ± 4.0	<0.001	0.001
BMI, %				<0.001	0.001
<18.5 kg/m^2	4.2	3.0	6.0		
18.5 to <25 kg/m^2	55.7	49.2	65.1		
25 to <30 kg/m^2	30.6	35.4	23.7		
>30 kg/m^2	9.5	12.4	5.3		
Household composition, %					
Couple with at least one child	31.7	28.2	36.8	<0.001	
Couple with no child	42.3	49.3	32.0		
Single with at least one child	5.4	4.2	7.0		
Single with no child	20.6	18.3	23.9		
Missing information	0.1	.	0.2		
Socio-professional status, %				<0.001	
Active	56.2	52.6	61.5		
Unemployed	4.2	3.4	5.3		
Student	4.9	1.6	9.6		
Retired	25.9	34.3	13.8		
Homemaker	8.8	8.1	9.8		
ICU, euros/month [3]	1328 ± 837	1359 ± 830	1285 ± 847	0.069	
Current smoking status, %				<0.001	0.0216
Smoker	27.9	23.7	34.0		
Non-smoker	70.3	74.8	63.7		
Missing	1.8	1.5	2.3		
IPAQ, %				0.156	0.239
Low	22.6	20.9	25.1		
Moderate	30.5	31.2	29.6		
High	45.7	46.8	44.0		
Missing information	1.2	1.1	1.3		
Screen for leisure time, minutes/day [3,4]	205 ± 138	195 ± 128	221 ± 151	0.002	0.020
In front of computer	60 ± 97	52 ± 94	73 ± 101	<0.001	0.249
In front of TV	145 ± 98	143 ± 89	148 ± 110	0.442	0.040
Frequency of eating between meals, as declared %				<0.001	<0.001
≥4 times/day	2.4	1.3	4.0		
2 to 3 times/day	15.1	11.8	19.8		
1/day	31.5	28.1	36.3		
>0 and <1/day	25.3	26.6	23.4		
Never	23.4	29.6	14.4		
Missing/invalid answers	2.4	2.6	2.1		
Interest in diet, %				0.001	0.010
A lot	32.8	36.4	27.7		
Little	44.7	44.6	45.0		
Not really	16.3	13.3	20.7		
Not at all	4.9	4.5	5.3		
Missing/invalid answers	1.3	1.2	1.3		

Abbreviations: BMI, body mass index; ICU, income per consumption unit; IPAQ, International Physical Activity Questionnaire. [1] p value provided by chi-squared test for categorical variables and GLM for continuous variables; [2] Gender-age adjusted p values provided by logistic regression for categorical variables and GLM for continuous variable; [3] Results are Mean ± SD; [4] One missing information items for BMI and seven missing information items for screen for leisure time variable.

Physical activity (IPAQ) level did not significantly differ between groups. However, the FS-EXCESS individuals spent significantly more time sitting in front of computers or television (+25 min per day) than the FS-ACCEPTABLE individuals, but this difference between groups was no longer significant after adjustment for age and gender. The FS-EXCESS individuals declared that they ate more often

between meals, and they were less interested in their diet; these results remained significant after adjustment for age and gender.

3.2. Observed Nutritional Intakes and Diet Quality Indicators

Observed nutritional intakes and diet quality indicators are detailed in Table 2. Compared with the FS-ACCEPTABLE group, individuals in the FS-EXCESS group had higher daily energy intakes (2192 vs. 2123 kcal/day), with a higher energy contribution of carbohydrates (45.4% energy vs. 40.8%) and lower energy contributions from proteins and fats (respectively 15.3% and 37.1% vs. 17.3% and 39.4%) after adjustment for age, gender and energy intake (except for energy intake, only adjusted for age and gender) or further adjustment for other sociodemographic and lifestyle parameters (see footnote to Table 2 for details). With all adjustments, energy intakes at main meals did not significantly differ between the two groups, unlike energy intakes at snacking occasions, higher in FS-EXCESS vs. FS-ACCEPTABLE groups (258 kcal/day vs. 131 kcal/day). The quantity of free sugars at each moment of consumption (meals or snacking occasion) was higher in FS-EXCESS vs. FS-ACCEPTABLE individuals. For FS-ACCEPTABLE individuals, the quantity of free sugars consumed in main meals was 4.3 times greater than at snacking occasions, while this ratio was only 2.6 for the FS-EXCESS group (data not shown). Compared with FS-ACCEPTABLE individuals, those in the FS-EXCESS group ate a more energy-dense diet (185 versus 165 kcal/100 g), and had lower nutritional quality diets, as shown by a lower PANDiet score, a lower MAR and a higher MER. For 11 out of the 23 nutrients of the MAR, capped percentages of recommended intakes were lower for FS-EXCESS group compared with FS-ACCEPTABLE group. There were no significant differences for the 12 remaining nutrients. When looking at the MER, among the three nutrients, free sugars were driving the difference between the two FS groups (Table S2).

Table 2. Observed nutritional intakes and diet quality indicators for the total sample and for FS-ACCEPTABLE and FS-EXCESS groups (mean ± SD).

	ALL	FS-ACCEPTABLE	FS-EXCESS	p^1	p^2	p^3
Individuals, *n*	1693	1003	690			
		Mean ± SD				
Energy intake (kcal/day) [4]	2151 ± 536	2123 ± 539	2192 ± 529	0.016	0.007	0.008
from main meals (kcal/day)	1969 ± 501	1992 ± 509	1935 ± 487	0.158	0.316	0.359
from snacking occasions (kcal/day)	183 ± 194	131 ± 154	258 ± 220	<0.001	<0.001	<0.001
Proteins, % of energy	16.5 ± 2.7	17.3 ± 2.7	15.3 ± 2.2	<0.001	<0.001	<0.001
Fats, % of energy	38.5 ± 5.7	39.4 ± 6.0	37.1 ± 4.8	<0.001	<0.001	<0.001
Carbohydrates, % of energy	42.7 ± 6.1	40.8 ± 6.2	45.4 ± 5.0	<0.001	<0.001	<0.001
Free sugars, % of energy	9.5 ± 5.1	6.3 ± 2.5	14.2 ± 4.2	<0.001	<0.001	<0.001
Starch, g/day	141.1 ± 51.2	143.5 ± 55.7	137.7 ± 43.6	<0.001	<0.001	<0.001
Total sugars, g/day	90.2 ± 37.3	75.1 ± 29.2	112.1 ± 37.1	<0.001	<0.001	<0.001
Free sugars g/day	51.9 ± 33.1	33.5 ± 16.6	78.7 ± 33.1	<0.001	<0.001	<0.001
from main meals (g/day)	39.4 ± 24.5	27.2 ± 14.4	57.0 ± 25.5	<0.001	<0.001	<0.001
from snacking occasions (g/day)	12.6 ± 16.3	6.3 ± 7.2	21.7 ± 20.9	<0.001	<0.001	<0.001
Non-free sugars, g/day	38.3 ± 19.6	41.6 ± 21.1	33.4 ± 16.0	<0.001	<0.001	<0.001
Alcohol, g/day	0.22 ± 0.74	0.18 ± 0.63	0.27 ± 0.87	0.086	0.052	0.050
Solid energy density, kcal/100 g	173.4 ± 32.7	165.2 ± 14.9	185.3 ± 31.6	<0.001	<0.001	<0.001
Variety, number of foods/week	58.4 ± 14.9	57.4 ± 7.6	60.0 ± 14.7	0.017	0.006	0.014
PANDiet	62.7 ± 7.5	64.3 ± 12.8	60.4 ± 6.6	<0.001	<0.001	<0.001
MAR, %	83.8 ± 9.0	84.8 ± 23.9	82.4 ± 9.3	<0.001	<0.001	<0.001
MER, %	32.2 ± 30.0	25.1 ± 14.9	42.6 ± 34.6	<0.001	<0.001	<0.001

Abbreviations: PANDiet, probability of adequate nutrient intake; MAR, mean adequacy ratio; MER, mean excess ratio. [1] GLM with survey design adjusted for age, gender and energy intake (except for energy intake, adjusted for age and gender only); [2] GLM with survey design adjusted for age, gender, energy intake, smoking status, BMI and socio-professional status (except for energy intake, adjusted for age and gender only); [3] GLM with survey design adjusted for age, gender, energy intake, smoking status, BMI, socio-professional status, composition of the family and sitting time (except for energy intake, adjusted for age and gender only); [4] 1 kcal =4.184 kJ.

3.3. Food Amounts in Observed Diets

Food amounts in the observed diets of FS-ACCEPTABLE and FS-EXCESS groups are detailed in Table 3. The amounts of fruits, vegetables, starchy foods (except ready-to-eat cereals), meat/eggs/fish, cheese, water and added fats were higher in the FS-ACCEPTABLE than in the FS-EXCESS group. By contrast, the amounts of sweet products (all sub-categories), sugar-sweetened beverages, fruit juices and sweet yogurts were higher in the FS-EXCESS than in the FS-ACCEPTABLE group. All these differences were significant after adjustment for all the variables considered, except for water (significantly different between groups after adjustment for age, gender and energy intake only).

3.4. Food Amounts and Weight Variations after Optimization

Food amounts in optimized diets are given in Table 3, and food weight variations between observed and optimized diets (i.e., dietary changes induced by the optimization process) are shown in Figure 1.

At the food category level (Figure 1A), for both FS-ACCEPTABLE and FS-EXCESS individuals, the optimization process significantly increased the amount of fruits/vegetables/nuts and starchy foods, and significantly decreased the amount of meats/eggs/fish, mixed dishes/sandwiches and added fats (all p values < 0.001 except for added fats in FS-EXCESS, $p = 0.012$). The amount of dairy products and beverages was significantly increased for the FS-ACCEPTABLE individuals only, while sweet products were decreased for the FS-EXCESS individuals only (all p values < 0.001). The other changes at food category level were not significantly different from 0.

At the sub-category level, for both FS-ACCEPTABLE and FS-EXCESS individuals, fresh fruits (Figure 1B) and both refined and unrefined starchy foods (Figure 1C) were increased. The amount of vegetables was increased only for FS-EXCESS individuals (Figure 1B). Plain yogurts significantly increased and cheese decreased for both groups, whereas plain milk and sweet yogurts increased significantly only for FS-ACCEPTABLE individuals (Figure 1D). All sub-categories of sweet products were decreased for FS-EXCESS individuals (Figure 1E). For the beverage category (Figure 1F), water and hot beverage sub-categories were significantly increased for both FS-ACCEPTABLE and FS-EXCESS, whereas sugar-sweetened beverages and fruit juices were decreased only for FS-EXCESS individuals.

3.5. Identification of the Most Binding Nutrients

Based on dual values, the most binding constraints (in decreasing order) were those on total energy, the maximal amounts of sodium, free sugars and saturated fatty acids and the minimal amount of total carbohydrates. They presented non-null dual values for more than 75% of individuals in the total sample (data not shown).

3.6. Changes in Sugar Balance after Optimization

The amounts of total, free and non-free sugars in the observed and optimized diets (g/day), from main food category contributors are shown in Figure 2 for FS-ACCEPTABLE and FS-EXCESS individuals.

For FS-ACCEPTABLE individuals, total sugars were significantly increased after optimization (+17.5 g/day) resulting from an increase in non-free sugars (+18.7 g/day), mainly due to an increase in fresh fruits (+16 g/day) and dairy products (+1.6 g/day) and a small decrease in free sugars (−1.2 g/day) from sweet products and beverages.

For FS-EXCESS individuals, to reach the maximum 10% energy from free sugars allowed by the model, the optimization significantly reduced free sugars (−25.5 g/day) through a decrease in sweet products (−14.3 g/day), sugar-sweetened beverages (−7.8 g/day) and fruit juices (−2.6 g/day) (Figure 2 and Table 3). Non-free sugars were significantly increased (+22.1 g/day), mainly due to an increase in fresh fruits (+19.5 g/day), in the fruits/vegetables/nuts category. All these changes led to a slight but significant decrease in total sugars (−3.4 g/day).

Table 3. Amounts of food categories and sub-categories in observed and optimized diets for the total sample and for FS-ACCEPTABLE and FS-EXCESS groups (g/day).

	Observed Diets				Optimized Diets				FS-Acceptable	FS-Excess
	ALL	FS-ACCEPTABLE	FS-EXCESS	p^1	ALL	FS-ACCEPTABLE	FS-EXCESS	p^1	p^2	p^2
Individuals, *n*	1693	1003	690		1693	1003	690			
		Mean ± SD				**Mean ± SD**				
Fruits/vegetables/nuts	379.7 ± 236.7	438.0 ± 248.1	294.8 ± 189.8	a,b,c	562.3 ± 177.2	583.8 ± 184.3	531.1 ± 161.4	a,b,c	<0.001	<0.001
Fruits	163.1 ± 148.8	190.8 ± 162.9	122.9 ± 114.1	a,b,c	322.3 ± 138.9	336.1 ± 144.9	302.2 ± 127.1	a,b,c	<0.001	<0.001
Fresh	149.6 ± 144.4	178.5 ± 159.1	107.4 ± 106.6	a,b,c	307.0 ± 137.2	321.8 ± 143.5	285.4 ± 124.5	a,b,c	<0.001	<0.001
Processed	13.6 ± 31.7	12.3 ± 31.1	15.5 ± 32.4	a,b,c	15.3 ± 31.9	14.3 ± 32.8	16.8 ± 30.4		<0.001	0.063
Vegetables	214.5 ± 142.3	245.2 ± 149.6	169.8 ± 117.4	a,b,c	234.6 ± 99.4	242.3 ± 102.8	223.4 ± 93.2		0.593	<0.001
Nuts	2.1 ± 5.8	2.0 ± 5.7	2.1 ± 6.0		5.4 ± 9.2	5.4 ± 9.3	5.5 ± 9.1		<0.001	<0.001
Starchy foods	254.2 ± 118.9	270.0 ± 127.1	231.2 ± 101.6	a,b,c	366.7 ± 109.4	375.4 ± 112.2	354.1 ± 104.1	a,b,c	<0.001	<0.001
Refined	168.8 ± 96.4	181.2 ± 102.5	150.7 ± 83.5	a,b,c	222.9 ± 92.2	229.1 ± 94.2	214.0 ± 88.4	a,b,c	<0.001	<0.001
Unrefined	80.5 ± 59.4	85.1 ± 64.6	73.9 ± 50.1	a,b,c	139.3 ± 56.6	142.7 ± 58.1	134.3 ± 53.8	a,b,c	<0.001	<0.001
Ready-to-eat cereals	4.9 ± 16.3	3.6 ± 13.4	6.7 ± 19.6		4.5 ± 14.2	3.6 ± 13.1	5.9 ± 15.5		0.881	0.033
Meats/eggs/fish	166.9 ± 70.2	179.9 ± 71.7	147.9 ± 63.4	a,b,c	142.8 ± 43.2	144.5 ± 42.2	140.2 ± 44.7		<0.001	0.001
Mixed dishes and sandwiches	120.9 ± 91.4	117.4 ± 93.7	125.9 ± 87.9		70.4 ± 53.8	65.1 ± 51.9	78.1 ± 55.6		<0.001	<0.001
Dairy products	209.4 ± 173.7	201.5 ± 174.4	221.0 ± 172.1		222.7 ± 146.0	222.7 ± 142.1	222.7 ± 151.6		<0.001	0.754
Milk	94.4 ± 151.6	85.5 ± 153.1	107.5 ± 148.4		109.7 ± 138.0	106.7 ± 133.2	114.1 ± 144.7		<0.001	0.183
Plain milk	86.8 ± 145.8	80.9 ± 151.6	95.5 ± 136.5		104.0 ± 134.3	102.9 ± 132.9	105.7 ± 136.4		<0.001	0.034
Sweet milk	7.6 ± 38.2	4.6 ± 28.1	12.0 ± 49.0		5.7 ± 29.3	3.8 ± 23.7	8.3 ± 35.8		0.202	0.004
Yogurts	80.9 ± 81.3	78.9 ± 79.9	83.7 ± 83.1		94.7 ± 83.6	95.8 ± 83.3	93.2 ± 84.0		<0.001	0.000
Plain yogurts	39.5 ± 60.5	44.8 ± 67.0	31.7 ± 48.6		48.1 ± 66.1	54.7 ± 71.6	38.7 ± 55.9		<0.001	<0.001
Sweet yogurts	41.4 ± 59.5	34.1 ± 53.4	52.0 ± 66.0	a,b,c	46.6 ± 61.4	41.1 ± 58.4	54.5 ± 64.8		<0.001	0.227
Cheese	34.1 ± 28.8	37.1 ± 29.8	29.8 ± 26.9	a,b,c	18.3 ± 14.0	20.2 ± 14.5	15.5 ± 12.9		<0.001	<0.001
Sweet products	119.9 ± 76.0	91.1 ± 58.7	161.7 ± 79.2	a,b,c	103.4 ± 65.5	91.3 ± 62.5	121.2 ± 65.7	a,b,c	0.907	<0.001
Milk or eggs-containing desserts	18.4 ± 31.0	12.3 ± 21.7	27.3 ± 39.3	a,b,c	17.4 ± 29.4	13.1 ± 23.9	23.6 ± 35.2	a,b,c	0.082	<0.001
Cakes and pastries	48.3 ± 44.7	41.1 ± 38.5	58.9 ± 50.6	a,b,c	39.1 ± 37.1	37.7 ± 36.3	41.0 ± 38.1		0.006	<0.001
Biscuits	8.2 ± 18.4	4.6 ± 10.8	13.6 ± 24.9	a,b,c	7.9 ± 15.3	5.8 ± 12.5	10.8 ± 18.1	a,b,c	<0.001	0.001
Croissants	17.5 ± 26.0	14.2 ± 23.6	22.1 ± 28.5	a,b,c	15.7 ± 24.3	15.3 ± 24.5	16.4 ± 24.0		0.267	<0.001
Confectionery (incl. chocolate)	5.8 ± 14.1	3.1 ± 6.4	9.9 ± 20.0	a,b,c	4.4 ± 7.9	3.2 ± 6.2	6.0 ± 9.6	a,b,c	0.229	<0.001
Honey, marmalade and chocolate spread	13.8 ± 19.8	10.4 ± 15.8	18.6 ± 23.8	a,b,c	13.5 ± 17.5	11.3 ± 16.3	16.7 ± 18.7	a,b,c	0.009	0.003
Table sugar	7.8 ± 10.2	5.5 ± 7.6	11.3 ± 12.4	a,b,c	5.5 ± 6.9	4.8 ± 6.7	6.7 ± 7.0	a,b,c	<0.001	<0.001
Beverages including water	1322.6 ± 630.3	1288.5 ± 655.7	1372.1 ± 588.3		1398.4 ± 403.0	1398.1 ± 421.3	1398.8 ± 374.9		<0.001	0.259
Water	798.6 ± 569.3	821.1 ± 596.0	765.7 ± 526.7	a	898.4 ± 412.1	911.2 ± 426.8	879.8 ± 389.3		<0.001	<0.001
Hot beverages	389.4 ± 335.3	403.7 ± 332.1	368.4 ± 339.0		412.4 ± 334.3	428.4 ± 325.0	389.1 ± 346.2		<0.001	<0.001
Diet beverages	12.9 ± 62.2	10.2 ± 58.3	16.9 ± 67.3		17.6 ± 72.2	14.6 ± 69.2	21.9 ± 76.3		0.018	0.009
Sugar-sweetened beverages	62.4 ± 175.4	17.0 ± 46.4	128.3 ± 255.5	a,b,c	23.5 ± 60.8	10.1 ± 31.1	43.1 ± 83.9	a,b,c	<0.001	<0.001
Fruit juices 100%	59.4 ± 89.9	36.4 ± 62.2	92.8 ± 111.4	a,b,c	46.5 ± 67.2	33.7 ± 55.0	65.0 ± 78.4	a,b,c	0.012	<0.001
Added fats and sauces	45.5 ± 23.4	48.1 ± 23.3	41.8 ± 23.1	a,b,c	39.4 ± 18.4	39.2 ± 19.0	39.8 ± 17.6		<0.001	0.012
Foods based on soya	3.5 ± 25.4	4.0 ± 25.9	2.9 ± 24.7		3.8 ± 25.5	4.0 ± 25.4	3.5 ± 25.6		0.907	0.168

Abbreviations: FS, free sugars; incl. chocolate, including chocolate. [1] Letters indicate a significant ($p < 0.01$) difference between FS-ACCEPTABLE and FS-EXCESS based on GLM with survey design at three levels of adjustments: a, GLM adjusted for age, gender and energy intake; b, GLM adjusted for age, gender, energy intake, smoker status, BMI and socio-professional status; c, GLM adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time; [2] GLM to test differences in dietary intakes between observed and optimized diets, with survey design adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time.

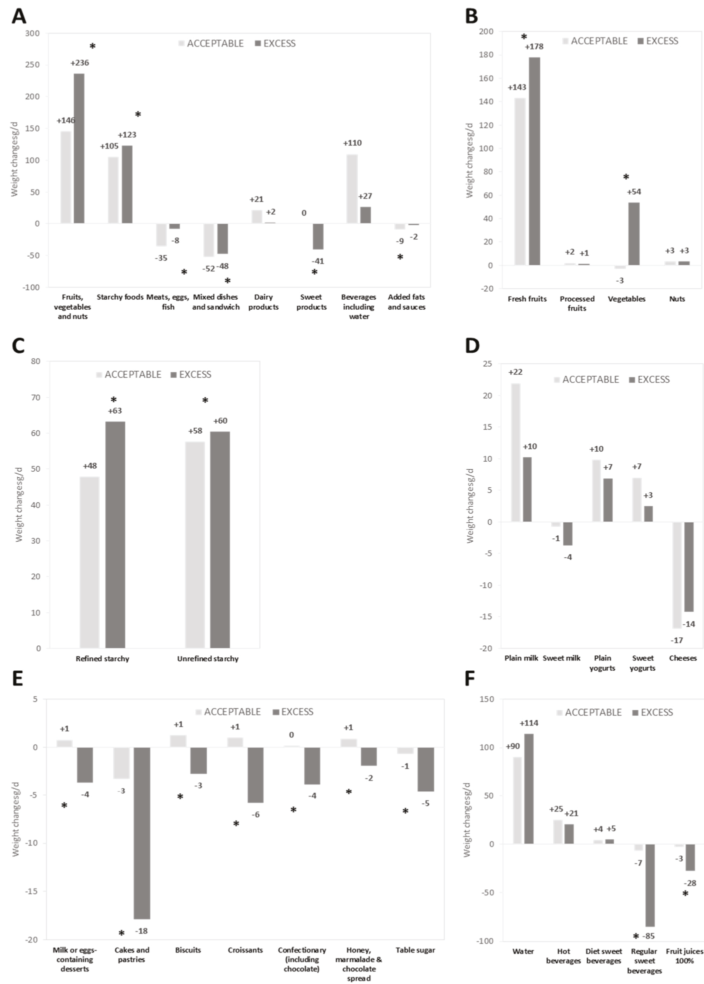

Figure 1. Weight changes [1] between observed and optimized diets (g/day) in food categories (**A**); and in food sub-categories for: fruits/vegetables/nuts (**B**); starchy foods (**C**); dairy products (**D**); sweet products (**E**); and beverages including water (**F**), in FS-ACCEPTABLE and FS-EXCESS individuals [2]. [1] Italic and bold values indicate a weight change significantly different from zero adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time; [2] the * symbol means that the weight changes were significantly different between FS-ACCEPTABLE and FS-EXCESS groups, adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time.

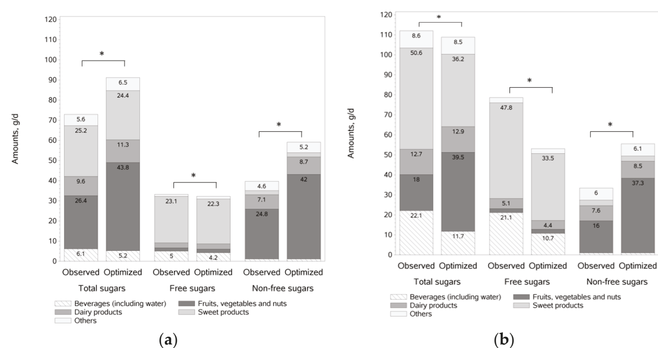

Figure 2. Amount of sugars (total, free, non-free) [1] in observed and optimized diets (g/d), from main food category contributors in: FS-ACCEPTABLE (**a**); and FS-EXCESS (**b**) individuals [2]. [1] Amounts of sugars lower than 4 g not labeled; [2] For both FS-ACCEPTABLE and FS-EXCESS, total sugars, free sugars and non-free sugars were significantly different between observed and optimized diets after adjustment for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time.

4. Discussion

In this representative sample of French adults, individuals with excessive intakes of free sugars represented 41% of the total population. Compared to the diets of individuals with acceptable free sugars intakes, their diets were found to be of lower nutritional quality, but could be optimized mostly via an increase in fresh fruits, vegetables and starchy foods, and a decrease in sweet products and sweet beverages including sugar-sweetened beverages and fruit juices.

Free sugar intakes represented 9.5% of energy intake in the French adult population. This is one of the lowest levels estimated by the WHO in European countries, and just below the WHO cut-off of 10%. Even so, individuals with free sugar intakes above this cut-off value (i.e., the FS-EXCESS group) represented 41% of the French adult population. Despite the existence of national and international recommendations on free sugars [9,11], it is currently difficult to estimate intakes of free sugars accurately, because information from nutrient composition tables is insufficient. Published studies on sugar intakes are mostly based on data on total and added sugars [13,15–17]. To our knowledge, only one other study recently conducted in the Dutch population [37] has also estimated free sugar intakes in a nationally representative sample. Compared with our French sample, a higher free sugar contribution (around 13.5% vs. 9.5% of energy intake) and with a lower adherence to the 10% WHO guidelines (around 30% vs. 59% respectively) was found in Dutch adults [37]. Interestingly, within the Dutch adult population, free sugar intakes decreased with age (from 16% to 11% of energy intake), in line with our findings, with FS-EXCESS individuals being 10 years younger than FS-ACCEPTABLE individuals.

Overall, in our sample, FS-EXCESS individuals had lower quality diets than FS-ACCEPTABLE individuals, as shown by a more energy-dense diet, lower MAR and PANDiet scores, and higher MER. These results are consistent with the conclusions of a recent review indicating that higher intake of added sugars is associated with poorer diet quality (in 20 out of 21 studies) and lower micronutrient intakes (in 21 out of 30 studies) [8]. Our results can be explained by food choices characterized by a lower consumption of foods of higher nutritional quality (e.g., fruits and vegetables) and a higher consumption of foods of lower nutritional quality (e.g., sweet products and sugar-sweetened beverages) [38] in FS-EXCESS individuals than in FS-ACCEPTABLE individuals.

FS-EXCESS individuals had a lower BMI than FS-ACCEPTABLE ones, despite higher energy intakes (+70 kcal/day) and greater sedentariness. A similar counterintuitive inverse relation between sugar intake and BMI was previously reviewed and discussed [39]. Selective underreporting of high sugar foods and drinks by overweight and obese people was listed as a potential explanatory factor.

The present survey being based on cross-sectional data, unhealthier eating patterns and lifestyles observed in FS-EXCESS individuals, may lead over time to weight gain, the extent of which could be estimated through the use of simplified dynamic energy balance models [40].

The top three food contributors to free sugar intakes were the same for both FS-ACCEPTABLE and FS-EXCESS groups, but with different contribution levels (measured in g/day of free sugars): sweet products (23.1 g/day and 47.8 g/day for FS-ACCEPTABLE and FS-EXCESS respectively) followed by beverages (5.0 g/day and 21.1 g/day) and dairy products (2.5 g/day and 5.1 g/day). Looking at food changes needed to achieve nutrient adequacy, the optimized diets showed similarities in the FS-ACCEPTABLE and FS-EXCESS groups (increase in fresh fruits, starchy foods, water, hot beverages and plain yogurts; decrease in mixed dishes/sandwiches, meat/eggs/fish and cheese). Additional food changes were found only in FS-EXCESS individuals, and consisted in a decrease in sweet products, sugar-sweetened beverages and fruit juices. Overall, the models were aimed not only at reducing free sugars, but also at ensuring a broad set of 33 nutritional recommendations were met without changing the energy level and thereby promoting nutrient density. This explains why food sub-categories containing free sugars were not necessarily decreased after optimization. For example, sweet yogurts were significantly increased in the FS-ACCEPTABLE individuals (+70 g/week). In other words, despite their free sugar content, the ID model selected sweet yogurts as a source of nutrients to favor.

Overall, the dietary changes needed to achieve nutrient adequacy were in line with the ongoing PNNS, the French national nutrition and health program (PNNS: "Programme National Nutrition Santé"), designed to improve health by helping people eat a healthier diet [41], where particular emphasis was placed on encouraging fruit and vegetable consumption, physical activity, and the consumption of whole grains, while reducing the consumption of foods with added sugars. In the US, to meet nutrient needs within calorie limits, advice has been recently given to choose a variety of nutrient-dense foods across and within all food groups, to limit calories from added sugars and saturated fats and to reduce sodium intake [12].

The strength of the present study lies in the ability to identify and quantify the dietary changes that may help any individual meet all 33 nutrient recommendations at the same time. Diet modeling with linear programming was early described as a unique tool to help develop food based dietary guidelines and public health messages [42]. Recently, the mean UK population diet was optimized to design the new Eatwell guide [43]. In the present study, individual diet modeling was used, rather than population diet modeling, in order to take into account the variability of individual food consumption. In addition, it is only with individual diet modeling that statistical analyses can be performed, therefore providing more robust conclusions. For FS-EXCESS individuals, dietary changes would mean halving sugar-sweetened beverages and fruit juices (from one glass/day to 1/2 glass/day) and table sugar (from 2 teaspoons/day to one teaspoon/day), reducing by about 1 portion /week for cakes and pastries, whereas fresh fruits would have to be greatly increased, with the addition of two portions of 80 g per day. If some of these changes could be difficult to integrate in daily life, our results do not seem to be drastically different from advice from a Register Dietician (RD). The RD would probably focus on the few major food changes able to rebalance the diet. The objective would not be to achieve adequacy for all nutrients at the same time but to correct major mistakes related to excesses or deficiencies. Overall, where a step-by-step approach would be taken by the RD, all the changes are considered at the same time with our mathematical model. Today, both approaches could be considered as complementary.

The present study has limitations. The choice was made to exclude alcoholic beverages from the present analyses because nutrient recommendations usually apply to non-alcoholic energy intakes. Similar choices were previously made in diet modeling with linear programming studies [43,44]. However, this limitation would appear negligible, since free sugars contained in alcoholic beverages contributed to only 0.16% of total energy intakes in our population (data not shown). In addition, allowing alcoholic beverages as variables in individual diet models was recently reported as difficult to manage, because they can contribute to energy requirements and some essential nutrients

(e.g., B vitamins and iron) for some individuals, without supplying any detrimental nutrients (e.g., sodium and saturated fatty acids) [44]. After optimization, the increase in total sugars seen in the FS-ACCEPTABLE group could also be considered as a limitation. However, we believe this increase would not have negative health effects since it is related to an increase in non-free sugars, mainly coming from fresh fruits. Indeed, in the absence of starch and total sugars recommendations, as fruits are more nutrient dense than starch, they are increased in large amounts by the optimization process to help meeting both micro-nutrient recommendations and the minimum carbohydrate energy contribution, leading to an increase in total sugars. Finally, the modeled diets could be questioned in terms of acceptability and feasibility. Despite its recognized interest in public health and nutrition [42], individual diet modeling with linear programming has only been used in the field of epidemiology. To improve realism, diets were optimized while staying as close as possible to current habits of each individual, such as preferentially using foods from his/her repertoire, which means that foods or drinks with lower nutritional profiles were not necessarily decreased or suppressed in the optimized diet [41]. For example, in the FS-ACCEPTABLE group, the amount of sweet products (91 g/day) did not change between observed and optimized diets. In terms of behavior change, adjusting food quantities rather than banning foods of low nutritional quality could be criticized, as some people may have difficulties keeping control over amounts consumed, highlighting the importance of considering individual psychological traits [45]. Future work could integrate complementary information on individual eating behavior characteristics, especially more refined acceptability parameters for a given individual. Generating results in portion sizes as well as integrating moments of consumption in the model would better target specific food changes in meals and snacking occasions. This would be particularly relevant for FS-EXCESS individuals studied here, as their dietary habits led to higher intakes of energy (in particular from sweet foods and drinks) specifically in snacking occasions compared with FS-ACCEPTABLE individuals. Once these model improvements have been made, these theoretical results will become more realistic, allowing some individual advice. Then, improvement of diet adequacy and metabolic parameters could be tested in a clinically relevant way.

5. Conclusions

In conclusion, the diet quality of French adults with excessive intakes of free sugars can be optimized by food changes that do not overly challenge their eating habits. To improve the estimation of free or added sugars and quality of food composition databases, initiatives such as the nutritional labeling of added sugars to be implemented on US food packages [46,47], are of interest to follow up. Finally, intervention studies are now needed to assess the feasibility, together with their short-term and long-term impact, of the changes in diet suggested by our study results.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/2/162/s1, Figure S1: Participant flow chart, Table S1: List of nutritional constraints included in the ID models, Table S2: Single nutrient ratios for MAR and MER for the total sample and for FS-ACCEPTABLE and FS-EXCESS groups (mean ± SD), Supplemental Methods: List of changes made to the previously published Individual Diet models.

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Author Contributions: A.L. contributed to the design of the study, interpreted the results, wrote the manuscript, and was responsible for the final content of the manuscript; M.M., R.G., F.V. conducted the study, performed the statistical analysis, interpreted the results and helped produce a final draft of the manuscript; F.D. contributed to the design of the study, interpreted the results, and helped produce a final draft of the manuscript; S.V. and N.D. contributed to the design of the study, interpreted the results, and wrote the manuscript; and all authors: read and approved the final version of the manuscript.

Conflicts of Interest: A.L., F.D. and S.V. are employees of Danone Nutricia Research. M.M., R.G., F.V. and N.D. declare no conflict of interest.

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Review

The Role of Carbohydrate Response Element Binding Protein in Intestinal and Hepatic Fructose Metabolism

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Abstract: Many articles have discussed the relationship between fructose consumption and the incidence of obesity and related diseases. Fructose is absorbed in the intestine and metabolized in the liver to glucose, lactate, glycogen, and, to a lesser extent, lipids. Unabsorbed fructose causes bacterial fermentation, resulting in irritable bowl syndrome. Therefore, understanding the mechanisms underlying intestinal and hepatic fructose metabolism is important for the treatment of metabolic syndrome and fructose malabsorption. Carbohydrate response element binding protein (ChREBP) is a glucose-activated transcription factor that controls approximately 50% of de novo lipogenesis in the liver. ChREBP target genes are involved in glycolysis (Glut2, liver pyruvate kinase), fructolysis (Glut5, ketohexokinase), and lipogenesis (acetyl CoA carboxylase, fatty acid synthase). ChREBP gene deletion protects against high sucrose diet-induced and leptin-deficient obesity, because *Chrebp*$^{-/-}$ mice cannot consume fructose or sucrose. Moreover, ChREBP contributes to some of the physiological effects of fructose on sweet taste preference and glucose production through regulation of ChREBP target genes, such as fibroblast growth factor-21 and glucose-6-phosphatase catalytic subunits. Thus, ChREBP might play roles in fructose metabolism. Restriction of excess fructose intake will be beneficial for preventing not only metabolic syndrome but also irritable bowl syndrome.

Keywords: carbohydrate response element binding protein; ChREBP; glycolysis; fructolysis; Glut5/SLC2A5; ketohexokinase; fructose

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1. Introduction

Obesity and its related diseases (diabetes mellitus, fatty liver, and dyslipidemia) are now significant social and economic problems in Western countries. A number of articles have discussed the relationship between fructose consumption (especially sugar-sweetened beverages) and the incidence of obesity and related diseases [1–5]. Increased fructose consumption contributes to the development of obesity accompanied by glucose intolerance, fatty liver, dyslipidemia, and hyperuricemia [3]. Additionally, in experimental animals, excess fructose intake causes body weight gain and fatty liver changes [3–5]. However, some studies have reported that there is no correlation between fructose consumption and obesity-related diseases [1,2]. Does fructose consumption really cause metabolic syndrome?

Plasma fructose levels (~200 μM in animals and 10–70 μM in humans) are much lower than plasma glucose levels (~6 mM) [6,7]. However, plasma fructose levels are positively correlated with glycemic control [7]. Fructose has more potent cytotoxicity because of increased advanced glycation end product (AGE) production [8,9]. Thus, fructose is not as readily absorbed and is immediately converted into other metabolites, such as glucose, triacylglycerol, and lactate in the intestine and liver [10–12]. If excess fructose is consumed, undigested fructose can cause bacterial fermentation, resulting in abdominal pain, flatulence, and diarrhea [13]. Therefore, clarification of the regulatory

mechanisms underlying intestinal and hepatic fructose metabolism will be beneficial for understanding the pathogenesis of not only obesity-related diseases, but also fructose malabsorption.

We have analyzed the role of carbohydrate response element binding protein (ChREBP) in the pathogenesis of metabolic diseases [14]. ChREBP is a glucose-activated transcription factor that regulates glucose and lipid metabolism [5,14–17]. ChREBP is abundantly expressed in the liver and intestine [18–20] and plays important roles in the regulation of fructose metabolism [20–22]. Moreover, ChREBP regulates the gene expression of proteins involved in monocarbohydrate transport, glycolysis, fructolysis, and de novo lipogenesis [20,23–25]. Therefore, ChREBP plays an important role in glycolysis and fructolysis. In this review, I describe glucose and fructose metabolism with special references to the roles of ChREBP. Fructose is slowly absorbed from the intestine and immediately metabolized in the liver. Considering the different roles between the liver and intestine, clarification of the mechanisms underlying both intestinal and hepatic fructose metabolism is important.

2. Metabolic Fate of Fructose

2.1. The Role of the Intestine in Fructose Metabolism

Fructose is a simple ketonic monosaccharide that is rich in fruits and honey. Fructose is used commercially in beverages for its high relative sweetness. Fructose is passively absorbed from the lower part of the duodenum and jejunum by glucose transporter 5 (GLUT5/SLC2A5) and transported into the blood by glucose transporter 2 (GLUT2/SLC2A2) [26,27]. Some studies have reported that the Michaelis constant (K_M) of SLC2A5 for fructose is ~6 mM and that of SLC2A2 is ~11 mM [12]. In the intestine, absorption rates for fructose are much slower than those for glucose [12]. In one study in humans, ingestion of 5 or 10 grams of fructose led to 10% of the study group being diagnosed as fructose malabsorbers [28]. This number increased to 40% when 20 grams of fructose was ingested [28]. Almost 40% of patients exhibited fructose malabsorption at an intake of 25 grams, and 66% of patients at an intake of 50 grams [29]. The absorption capacity of fructose in monosaccharide form in adult rats was equivalent to 1.4–1.6 g fructose/kg body weight [30]. Acute fructose malabsorption occurred with doses greater than 2.1–2.4 g/kg body weight [30]. Moreover, fructose malabsorption is caused by defects in fructose transporters, such as SLC2A5 and SLC2A2 [12]. Intestinal fructose malabsorption causes abdominal complaints, such as abdominal pain, bloating, flatulence, and diarrhea. These symptoms are due to bacterial fermentation of unabsorbed fructose in the colon. Deletion of the gene encoding *Slc2a5* in mice fed a high fructose diet resulted in decreased fructose absorption by 75% in the jejunum and decreased serum fructose levels by 90%. Similar to fructose malabsorption in humans, the caecum and colon in high fructose diet-fed *Slc2a5*$^{-/-}$ mice were dilated because of bacterial fermentation [31]. Thus, overconsumption of fructose causes irritable bowel syndrome in humans and animals.

2.2. Role of the Liver in Fructose Metabolism

Conversion from fructose into glucose is limited in intestine. At lower luminal fructose concentrations in the intestine (~1 mM), ~60% of fructose is converted to glucose [12]. At higher luminal concentrations, fructose is metabolized in the liver. Portal vein fructose concentrations are 1 mM, while peripheral fructose concentrations are ~0.1 mM [12]. As *SLC2A5* expression in the liver is much lower than in the intestine, fructose in the liver is transported by SLC2A2 and phosphorylated into fructose-1-phosphate by ketohexokinase (KHK)/Fructokinase [12]. Fructolysis is much faster than glycolysis. Enzymes specific for fructose metabolism include KHK, aldolase B, and triokinase (ATP: D-glyceraldehyde 3-phosphotransferase) (Figure 1). These enzymes are highly expressed in the liver, kidney, and intestine [32]. There are two KHK isoforms, KHK-C and -A. Both can metabolize fructose, but KHK-C is considered the primary enzyme involved in fructose metabolism because of its lower K_M [33–35]. In hepatocellular carcinomas, fructolysis is much slower than in healthy hepatocytes because of a switch from KHK-C to KHK-A [35]. Thus, KHK-C, rather than KHK-A, primarily regulates fructolysis. Fructolysis bypasses the steps using glucokinase and phosphofructokinase, which are rate-limiting

enzymes in glycolysis. Fructose-1-phosphate is then converted into dihydroxyacetone phosphate and glyceraldehyde via aldolase B. Glyceraldehyde is converted into glyceraldhyde-3-phosphate by triokinase. Dihydroxyacetone phosphate and glyceraldehyde-3-phosphate are identical to those in glycolysis and can enter the gluconeogenic pathway for glucose or glycogen synthesis or be further catabolized through the lower glycolytic pathway to lactate or de novo lipogenesis [11,12].

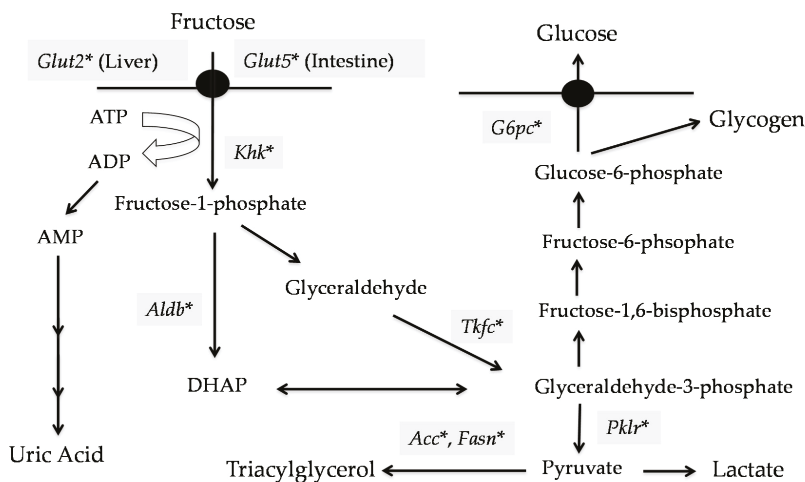

Figure 1. ChREBP regulates fructolytic gene expression. Fructose is transported by GLUT5 and metabolized by ketohexokinase, aldolase B, and triokinase. Dihydroxyacetone phosphate and glyceraldehyde-3-phosphate enter into the glycolytic or gluconeogenic pathway. * Genes are regulated by ChREBP [14,20,23–25]. *Khk*, ketohexokinase; *G6pc*, glucose-6-phosphatase catalytic subunit; *Aldb*, aldolase B; *Pklr*, pyruvate kinase, liver and reticulocyte type; *Acc*, acetyl coA carboxylase; *Fasn*, fatty acid synthase; *Tkfc*, triokinase; ChREBP, carbohydrate response element binding protein; GLUT2, glucose transporter 2; GLUT5, glucose transporter 5; DHAP, Dihydroxyacetone phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

In healthy subjects, after ingestion of a fructose load, plasma glucose and insulin levels change significantly less than those following a glucose load. Plasma fructose levels are increased to 50–500 µM [11]. Fructose is converted into glucose (28.9%–54%), lactate (~28%), glycogen (17%), and triacylglycerol (<1%) rapidly (<6 h) (Figure 2) [11]. These data suggest that the SLC2A5/SLC2A2-KHK system in the intestine and liver successfully protects against fructose toxicity. The contribution of excess fructose consumption to hyperlipidemia might be much lower in humans.

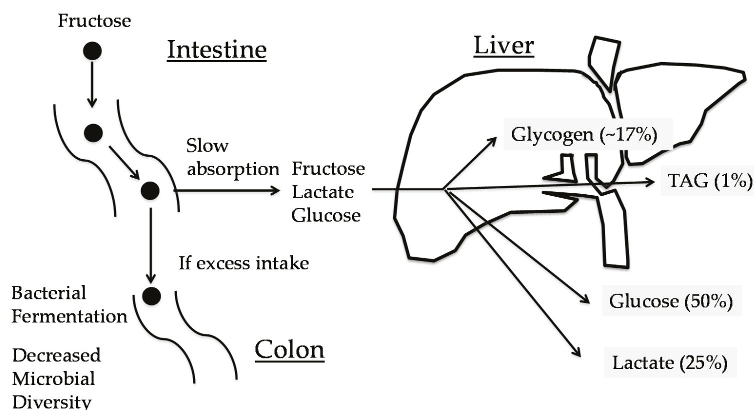

Figure 2. Metabolic fate of fructose. Fructose is slowly absorbed in the intestine. If excess fructose is consumed, unabsorbed fructose causes bacterial fermentation and, thereby, irritable bowel syndrome. Absorbed fructose is converted into glucose (50%), glycogen (~17%), lactate (25%), and triacylglycerol (TAG) (1%) [11].

3. ChREBP Regulates Glycolysis and Fructolysis through Altered Gene Expression

3.1. Glucose Metabolism

ChREBP is a transcription factor that belongs to a family of basic helix-loop-helix leucine zipper-type transcription factors [18,19]. ChREBP and Max-like protein X (MLX) form a heterodimer that binds carbohydrate response elements (ChoREs) in the promoters of ChREBP target genes [19,36,37]. ChREBP is expressed in the liver, kidney, intestine, muscle, white adipose tissue, brown adipose tissue, and pancreatic islets [18–20]. In contrast, Mlx is ubiquitously expressed across tissues [19].

ChREBP has two isoforms, ChREBP-α and ChREBP-β [38]. Both ChREBP isoforms and Mlx form complexes (ChREBP-α-MLX and ChREBP-β-MLX) that regulate ChREBP target gene expression [38]. ChREBP-α is less potent than ChREBP-β. However, ChREBP-α has a low glucose inhibitory domain [38]. Under low glucose conditions, the low glucose inhibitory domain suppresses ChREBP-α transactivity [39]. In contrast, ChREBP-β is constitutively active under any glucose conditions. ChREBP-β is induced by ChREBP-α [38], and ChREBP-β suppresses ChREBP-α expression [40–42]. Therefore, we hypothesized that ChREBP-α and ChREBP-β serve as a sensor and amplifier for glucose signaling, respectively [14]. The ChREBP-MLX complex regulates genes related to glycolysis, lipogenesis, gluconeogenesis, transcription factors, and hormone signaling [14,20,23–25]. Therefore, ChREBP contributes to glucose and lipid homeostasis by regulating metabolic gene expression.

ChREBP is activated by several metabolites, such as glucose-6-phosphate, xylulose-5-phosphate, fructose-2,6-bisphosphate, and Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), and suppressed by adenosine monophosphate (AMP), ketone bodies and cyclic cAMP [43–52] (Figure 3).

Metabolites that can activate ChREBP are involved in the glycolytic and pentose phosphate pathways [43–48]. Glycolysis and the pentose phosphate shunt are linked to de novo lipogenesis through nicotinamide adenine dinucleotide supply and demand [23]. Glycolysis (via the tricarboxylic acid cycle) and the pentose phosphate shunt supply the substrates citrate and the reduced form of nicotinamide adenine dinucleotide for de novo lipogenesis. ChREBP regulates genes involved in the glycolytic (genes encoding liver type pyruvate kinase and Glut2), pentose phosphate (gene encoding transketolase), and de novo lipogenic (genes encoding fatty acid synthase and acetyl CoA carboxylase) pathways (Figure 4). Thus, ChREBP plays an important role in regulating hepatic glycolytic and lipogenic gene expression.

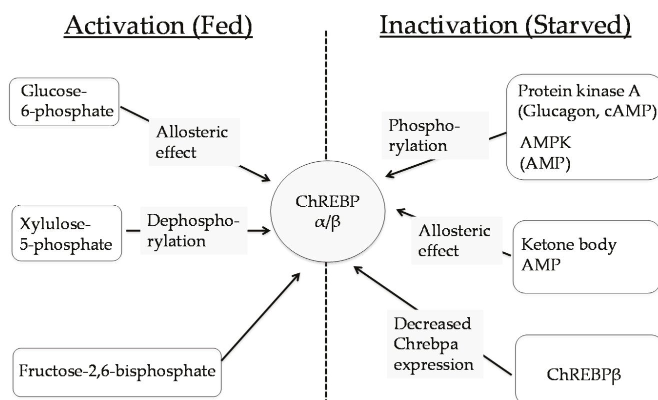

Figure 3. ChREBP transactivities are regulated by several factors. ChREBP is activated by glucose derived metabolites and suppressed by AMP, ketone bodies and cyclic cAMP [43–52]. AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; cAMP, cyclic AMP.

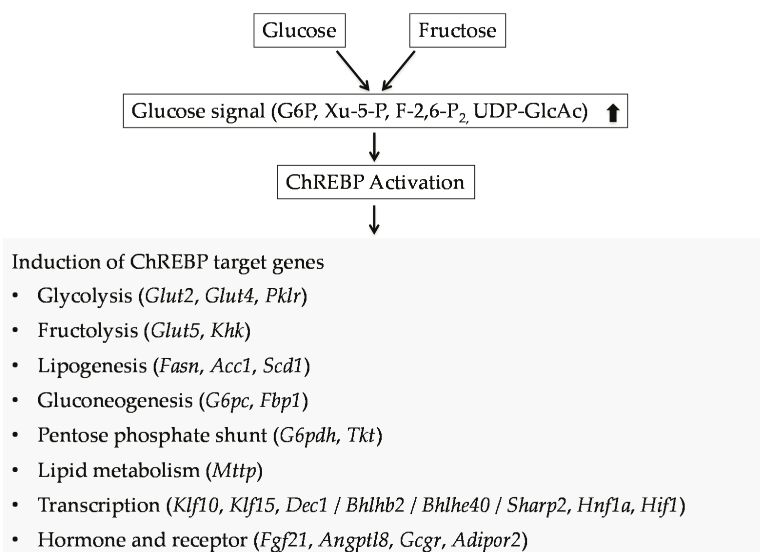

Figure 4. ChREBP has an important role in regulating glucose and lipid metabolism. Glucose and fructose regulate many genes expression through ChREBP activation [14,20,23–25]. *Glut2*, glucose transporter 2; *Glut4*, glucose transporter 4; *Pklr*, pyruvate kinase, liver and red blood cell; *Glut5*, glucose transporter 5; *Khk*, ketohexokinase; *Fasn*, fatty acid synthase; *Acc1*, acetyl coA carboxylase 1; *Scd1*, stearoyl CoA desaturase; *G6pc*, glucose-6-phosphatase catalytic subunit; *Fbp1*, fructose-1,6-bisphosphatase 1; *G6pdh*, hexose-6-phosphate dehydrogenase; *Tkt*, transketolase; *Mttp*, microsomal triglyceride transfer protein; *Klf10*, kruppel-like factor 10; *Klf15*, kruppel-like factor 15; *BHLHE40*, basic helix-loop-helix family, member E40; *Bhlhb2*, Basic helix-loop-helix domain-containing protein, class B; *Hnf1a*, hepatocyte nuclear factor 1a; *Hif1*, hypoxia inducible factor 1; *Fgf21*, fibroblast growth factor 21; *Angptl8*, angiopoietin like 8; *Gcgr*, glucagon receptor; *Adipor2*, adiponectin receptor 2.

3.2. Fructose Metabolism

As with genes related to fructose metabolism, glucose enhances fructose absorption in the intestine. Glucose and triiodothyronine coordinately induce *SLC2A5* mRNA expression in human colon CACO2 cells [53]. Similarly, fructose and triiodothyronine coordinately induce *Slc2a5* mRNA expression in the small intestine of rats during the weaning period [54]. However, in weaning pups made hypothyroid from birth, dietary fructose can still enhance intestinal fructose uptake and *Slc2a5* mRNA expression, even though thyroxine levels in the serum are very low. Therefore, glucose and fructose primarily activate *Slc2a5* mRNA expression in vivo and in vitro.

There are a few mechanisms underlying glucose- and fructose-induced *Slc2a5* mRNA expression. ChREBP is known to regulate *Slc2a5* gene expression. *Chrebp*$^{-/-}$ mice displayed lower *Slc2a5* mRNA levels in the intestine and liver than those in wild-type (WT) mice [20] (Iizuka K and Kato T, unpublished data). Similarly, glucose upregulates *Slc2a5* mRNA expression, and overexpression of dominant negative MLX suppresses glucose induction of *Slc2a5* mRNA in primary rat hepatocytes [23]. Similar to glucose, fructose might activate ChREBP transactivity by *O*-glycosylation (via the hexosamine pathway), phosphorylation (via xylulose-5-phosphate), and conformational change (via glucose-6-phosphate) [5,21,22,43–48]. Furthermore, fructose can increase *Slc2a5* mRNA stability through the cAMP pathway and polyadenylated-binding protein-interacting protein 2 binding [55].

Recently, some groups reported that there was an indirect pathway mediated by thioredoxin-interacting protein (TXNIP) [56,57]. TXNIP plays an important role in regulating intracellular redox state [58]. Glucose and fructose induce *TXNIP* gene expression partly through ChREBP and MondoA, an orthologue of ChREBP [59,60]. Fructose also promotes fructose uptake through the interaction between TXNIP and GLUT5/GLUT2. Consistent with this, *TXNIP* gene deletion prevented body weight gain and fatty liver caused by high fructose diet consumption.

KHK is also a gatekeeper gene that protects from increasing plasma fructose levels [5]. *Khka/c*$^{-/-}$ mice display fructosuria and decreased adiposity and hepatic fat content [31]. One group demonstrated that there are two ChoRE regions in human *KHK* promoters (proximal, −722 to −739 bp; distal, −2902 to −2885 bp) [61]. The ChoRE in the *Slc2a5* promoter is not yet identified. However, considering that *Slc2a5* and *Khk* gene deletion both suppress fructose-induced *Chrebp* gene expression [62], metabolites derived from fructose may regulate fructolytic gene expression through CHREBP activation (Figure 1).

4. Chrebp Deletion Suppresses Obesity and Fatty Liver Induced by Excess Carbohydrate Feeding

Does ChREBP regulate glucose and lipid homeostasis in vivo? The answer lies in the results from *Chrebp*$^{-/-}$ mice. *Chrebp*$^{-/-}$ mice display several characteristic phenotypes [20]. Compared with wild type (WT) mice, *Chrebp*$^{-/-}$ mice exhibit hepatomegaly because of hepatic glycogen accumulation and reduced white adipose tissue weights [20]. Additionally, plasma free fatty acid, ketone body, and cholesterol levels in *Chrebp*$^{-/-}$ mice were much lower than those in WT mice [20,63].

Ob/ob mice are characterized by a leptin gene mutation and display excess dietary intake. *Chrebp* gene deletion prevents body weight gain and fatty liver by decreasing food intake [64]. The results of adenoviral short hairpin ribonucleic acid (shRNA) against *Chrebp* in ob/ob mice were similar to our results [65]. Similarly, in *Chrebp*$^{-/-}$ mice fed a high fat/high cholesterol/high sucrose diet, body weight gain was suppressed because of decreased food intake [63]. These mice also displayed cholesterol gallstones (Iizuka K, unpublished data). In contrast, in *Chrebp*$^{-/-}$ mice fed a high starch diet, body weight gain was similar to that in WT mice [20]. In *Chrebp*$^{-/-}$ mice fed a high fat/low sucrose diet, similar results were also observed (Iizuka K and Takao K, unpublished data). These data indicate that *Chrebp*$^{-/-}$ mice could not consume sucrose or fructose. Moreover, high sucrose-fed *Chrebp*$^{-/-}$ mice displayed massive dilatation of the caecum and colon, similar to *Slc2a5*$^{-/-}$ mice fed a high sucrose diet (Iizuka K, et al. unpublished data) [30]. As *Slc2a5* mRNA is regulated by ChREBP, the inability of *Chrebp*$^{-/-}$ mice to consume sucrose might be partly due to decreased intestinal *Slc2a5* expression. We are now working to identify the mechanism underlying why *Chrebp*$^{-/-}$ mice could not consume a fructose-rich diet and, particularly, the role of the intestine.

5. Newly-Identified Roles of ChREBP: Regulation of Sweet Preference and Hepatic Glucose Production

5.1. Fibroblast Growth Factor (FGF)-21

FGF-21 is a promising therapeutic target for obesity and dyslipidemia [66]. FGF-21 is a secretory hormone induced by starvation through peroxisome proliferator-activated receptor alpha. In contrast, plasma FGF-21 levels in obese individuals are much higher those in lean individuals. We observed that ChREBP directly activated *Fgf-21* gene expression in rat hepatocytes [67]. Moreover, some studies have reported that oral glucose and fructose injections increase plasma FGF21 levels [68] and acute increase in circulating FGF-21 following fructose gavage was absent in ChREBP knockout mice [22]. In contrast, induction of ChREBP-β and its gene targets were attenuated in *Fgf-21*$^{-/-}$ mice fed high-fructose diets [22]. After eight weeks of high-fructose diet, livers from *Fgf-21*$^{-/-}$ mice demonstrate atrophy and fibrosis [22]. Considering that FGF-21 did not directly affect ChREBP transactivity in rat hepatocytes [68,69], probably the effect of FGf21 gene deletion on fructose induced hepatic fibrosis might be due to indirect pathway. Recently, some groups have reported that FGF-21 modulates simple sugar intake and sweet taste preference by producing an endocrine satiety signal that acts centrally to suppress sweet intake [70,71]. The liver-to-brain FGF21 axis may represent a negative feedback loop, as hepatic FGF21 production is elevated by glucose- and fructose-mediated ChREBP activation (Figure 5).

5.2. Glucose-6-Phosphatase Catalytic Subunit (G6pc)

The relationship between fructose consumption and hepatic insulin resistance has been documented [3]. *G6pc* mRNA expression is decreased in *Chrebp*$^{-/-}$ mice [20,21,64], and some studies have reported that ChREBP directly regulates *G6pc* gene expression in hepatocytes [72]. A recent report revealed that ChREBP regulated fructose-induced hepatic glucose production through increased *G6pc* expression [20]. Interestingly, in *Chrebp*$^{-/-}$ mice, glucagon failed to stimulate glycogenolysis and, thereby, glucose production [20]. Considering that ChREBP regulates glucagon receptor (*Gcgr*) gene [23,73], not only *G6pc* but also *Gcgr* has some role in ChREBP-mediated glucose production from frcutose. Moreover, they also reported that ChREBP-β is correlated with *G6pc* expression as well as expression of the genes encoding liver pyruvate kinase and fatty acid synthase in liver biopsy samples from overnight-fasted human subjects with non-alcoholic fatty liver disease [21,74].

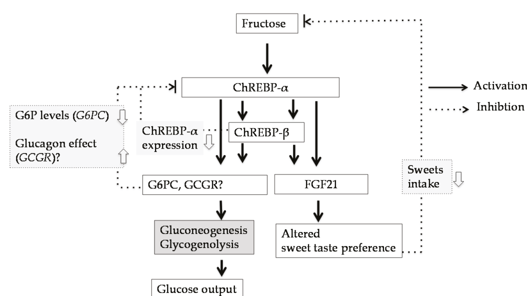

Figure 5. Fructose induces G6pc and Fgf21 gene expression thorugh ChREBP activation. ChREBP-α regulates ChREBP target genes expression. In turn, products of ChREBP target genes (ChREBP-β, G6pc, Gcgr, and Fgf-21) might suppress ChREBP transactivity [20–22,67,73]. FGF-21 suppress ChREBP transactivity by decreasing sweets intake [70,71]. GCGR might suppress ChREBP activity by enhancing glucagon effects and thereby protein kinase A activity. G6PC might suppress ChREBP activity by decreasing intracellular G6P levels. G6PC, glucose-6-phosphatase catalytic subunit; GCGR, glucagon receptor; FGF21, fibroblast growth factor-21; G6P, glucose -6-phosphate; ChREBP, carbohydrate response element binding protein.

However, there is now epidemiological controversy regarding fructose consumption and insulin resistance [1,2]. A fructose intake exceeding 150 g/day in adults reduces fasting insulin sensitivity, and fructose intake exceeding 250 g/day suppresses hepatic glucose output by insulin in humans [1]. When solutions containing 25–50 g of fructose (equivalent to >500 mL high fructose corn syrup-sweetened soft drink) are consumed, >50% of healthy subjects demonstrate fructose malabsorption and, consequently, experience symptoms of abdominal pain, flatulence, and loose bowels [26,27]. Considering the difficulty of intestinal fructose absorption, whether fructose-mediated ChREBP activation in the liver contributes to hepatic glucose output regulation should be further investigated (Figure 5).

6. The Role of ChREBP in Fructose Metabolism

As described above, slow fructose absorption from the intestine and faster conversion from fructose into glucose is important for fructose metabolism. If fructose absorption rates were as fast as glucose, and fructolysis was regulated by a negative feedback system, plasma fructose levels would be as high as plasma glucose levels. As fructose is more potent and much faster in terms of hemoglobin A1c (HbA1c) formation [8,9], HbA1c levels and diabetic vascular complications would worsen. Therefore, although fructose can theoretically induce metabolic syndrome in high fructose-fed animal models, intestinal fructose absorption might normally be a rate-limiting barrier that protects from increasing plasma fructose concentration. ChREBP potentially regulates both intestinal and hepatic fructose metabolism. ChREBP suppression is beneficial for metabolic syndrome and obesity, and several anti-dyslipidemic and anti-diabetic drugs are known to suppress ChREBP transactivity [14]. If these drugs (for example, metformin) impact the intestine, excess sucrose and fructose intake might cause diarrhea and abdominal pain because of decreased fructose absorption. Thus, irritable bowel syndrome may be a warning symptom to prevent against excess fructose intake. A diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols is known to be beneficial for the treatment of irritable bowl syndrome [75]. Therefore, restriction of excess fructose intake, such as fructose-containing beverages, and lowering consumption of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols will be beneficial for protecting against metabolic syndrome and irritable bowel syndrome.

7. Conclusions

ChREBP plays an important role in regulating fructose absorption and conversion from fructose into glucose, lactate, glycogen, and lipids. The role of ChREBP in fructose-mediated fatty liver might be very low because fructose is difficult to be absorbed in the intestine. However, chronic fructose intake might increase the efficiency of intestinal fructose absorption through intestinal Glut5 expression induced by ChREBP activation. Considering that fructose is harmful in the development of metabolic syndrome and irritable bowl syndrome, restriction of fructose intake might be important for protection against these conditions.

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Article

Fructose-Rich Diet Affects Mitochondrial DNA Damage and Repair in Rats

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Abstract: Evidence indicates that many forms of fructose-induced metabolic disturbance are associated with oxidative stress and mitochondrial dysfunction. Mitochondria are prominent targets of oxidative damage; however, it is not clear whether mitochondrial DNA (mtDNA) damage and/or its lack of repair are events involved in metabolic disease resulting from a fructose-rich diet. In the present study, we evaluated the degree of oxidative damage to liver mtDNA and its repair, in addition to the state of oxidative stress and antioxidant defense in the liver of rats fed a high-fructose diet. We used male rats feeding on a high-fructose or control diet for eight weeks. Our results showed an increase in mtDNA damage in the liver of rats fed a high-fructose diet and this damage, as evaluated by the expression of DNA polymerase γ, was not repaired; in addition, the mtDNA copy number was found to be significantly reduced. A reduction in the mtDNA copy number is indicative of impaired mitochondrial biogenesis, as is the finding of a reduction in the expression of genes involved in mitochondrial biogenesis. In conclusion, a fructose-rich diet leads to mitochondrial and mtDNA damage, which consequently may have a role in liver dysfunction and metabolic diseases.

Keywords: fructose-rich diet; mitochondrial biogenesis; mitochondrial DNA (mtDNA); oxidative damage; repair mechanisms

1. Introduction

Over the last few decades, the daily intake of fructose, either free or as high-fructose corn syrup, has markedly increased [1]. These forms of fructose are used in the food industry for their enhanced sweetness, palatability, solubility, lower cost, and high production efficiency compared to sugar [2]. However, the increase in fructose consumption has coincided with a rise in the incidence of obesity, metabolic syndrome, and type 2 diabetes [3,4]. Several researchers have even suggested that increased fructose consumption has directly contributed to the obesity and type 2 diabetes epidemic [3,4].

Previous studies have shown that fructose-rich diets can induce many features of metabolic syndrome, including hypertension, insulin resistance, abdominal obesity, hepatic steatosis, endothelial dysfunction, and inflammation [5–10]. Fructose is a highly lipogenic substrate which can induce profound metabolic alterations in the liver [11], where 90% of ingested sugar is metabolized [12]. Although the mechanisms underlying fructose-mediated metabolic disease are not entirely understood, previous studies have suggested a causative role for oxidative stress [13–16], an imbalance between

reactive oxygen species (ROS) generation and removal by antioxidant defense systems. Mitochondria produce ROS through the respiratory chain, but are also equipped with antioxidant enzymes, thereby participating in redox regulation. Since oxidative stress is considered a key factor in the development of metabolic alterations [17,18], the marked effect of fructose on systemic oxidative stress could explain its role in the pathophysiology of insulin resistance and metabolic syndrome [19–21]. In agreement with this, in rats fed a high-fructose diet, we have previously found hepatic insulin resistance together with hepatic mitochondrial oxidative damage, both in the lipid and in the protein component, as well as decreased activity of antioxidant defense [11]. In addition, Mamikutty et al. [22] found that after eight weeks of high fructose consumption, rats developed several features of metabolic syndrome, together with mitochondrial structural alterations. Therefore, it seems that there is a strong association between the effect of fructose at the cellular level and the mitochondrial compartment. However, information on the possible mechanism by which mitochondrial function is altered by fructose feeding is lacking. Mitochondria contain a double-stranded, circular DNA that encodes many proteins essential for ATP production. Malfunction of the antioxidant defense system leads to oxidative attack, resulting in mtDNA damage, which in turn can lead to a decline in mitochondrial functions and turnover, and in an impairment of appropriate stress responses that monitor and maintain their quality. Taking into account the above considerations, we believe that it becomes relevant to investigate the consequences that a fructose-rich diet may have on oxidative stress and on mtDNA damage in a metabolically very important organ such as the liver.

Therefore, the purpose of this study was to evaluate in the liver of fructose-fed rats: (i) a possible induction of mtDNA damage; (ii) changes in the expression of a specific enzyme associated with the mtDNA repair mechanism; and (iii) a possible involvement of mechanisms underlying mitochondrial biogenesis and the cellular antioxidant defense system.

2. Materials and Methods

2.1. Animals and Treatments

Male Sprague-Dawley rats (Charles River, Calco (LC), Italy) of about 100 days of age were caged singly in a temperature-controlled room (23 ± 1 °C) with a 12 h light/dark cycle (6:30 a.m.–6:30 p.m.). Animal treatment, housing, and euthanasia met the guidelines set by the Italian Health Ministry. All experimental procedures were also approved by "Comitato Etico-Scientifico per la Sperimentazione Animale" of the University "Federico II" of Naples, Italy.

Rats were divided in two groups, each with the same mean body weight (470 ± 10 g), and either fed a control or a fructose-rich diet (composition of the two diets is shown in Table 1), known to induce early signs of obesity within eight weeks of treatment [7,8,11]. Briefly, rats were pair-fed for eight weeks, by giving them the same amount of diet, both as weight and as caloric content and each rat consumed the full portion of the diet. During the treatment, body weight, food, and water intake were monitored daily. At the end of the experimental period, the rats were euthanized by decapitation, and blood and liver samples were collected.

2.2. Plasma Parameters

Plasma concentrations of alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured by colorimetric enzymatic method using commercial kits (SGM Italia, Rome, Italy).

On plasma samples, 8-hydroxy-2′-deoxyguanosine (8-OHdG), a critical biomarker of oxidative stress [23] was quantified using a DNA/RNA Oxidative Damage ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. Plasma samples were analyzed in duplicate. Standard 8-OHdG was assayed over a concentration range of 10.3–3000 pg·mL^{-1} in duplicate for each experiment.

Table 1. Composition of experimental diets.

Component (g 100 g^{-1})	Control Diet	Fructose Diet
Standard chow *	50.5	50.5
Sunflower oil	1.5	1.5
Casein	9.2	9.2
Alphacel	9.8	9.8
Starch	20.4	-
Fructose	-	20.4
Water	6.4	6.4
AIN-76 mineral mix	1.6	1.6
AIN-76 vitamin mix	0.4	0.4
Choline	0.1	0.1
Methionine	0.1	0.1
Gross energy density, KJ·g^{-1}	17.2	17.2
Protein, % metabolisable energy	29.0	29.0
Lipids, % metabolisable energy	10.6	10.6
Carbohydrates, % metabolisable energy	60.4	60.4
Of which: Fructose	-	30.0
Starch	52.8	22.8
Sugars	7.6	7.6

* Mucedola 4RF21; Italy.

2.3. Genomic DNA Isolation

Total liver DNA was extracted using the Genomic-tip 20/G kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The quantification of the purified genomic DNA and PCR products was performed fluorometrically using the Picogreen ds DNA reagent (Invitrogen, Milan, Italy).

2.4. Quantitative Polymerase Chain Reaction (QPCR)

QPCR was performed on liver DNA extracts as previously described [24] with the following modification: the PCR amplification was done using the Ranger DNA Polymerase with the appropriate premixes (Bioline Ltd., London, UK). Two pairs of PCR primers were employed:

mtDNA long fragment (13.4 Kbp): 5′-AAAATCCCCGCAAACAATGACCACCC-3′ (sense)/5′-GGCAATTAAGAGTGGGATGGAGCCAA-3′ (anti-sense);
mtDNA short fragment (235 bp): 5′-CCTCCCATTCATTATCGCCGCCCTGC-3′ (sense)/5′-GTCTGGGTCTCCTAGTAGGTCTGGGAA-3′ (anti-sense).

For amplification of the mtDNA long fragment, the standard thermocycler program included initial denaturation at 94 °C for 1 min, 18 cycles of 94 °C for 15 s, 65 °C for 12 min, and final extension at 72 °C for 10 min. To amplify the short mtDNA fragment (235 bp), the same program was used except the extension temperature was changed to 60 °C. DNA damage was quantified by comparing the relative efficiency of amplification of the long mtDNA fragment normalized to the amplification of the small mtDNA fragment. QPCR products were quantified using PicoGreen dye and a fluorescence plate reader in the same manner as the template DNA. The resulting values were converted to relative lesion frequencies per 10 Kbp DNA by applying the Poisson distribution.

2.5. mtDNA Copy Number

Relative mtDNA copy numbers were measured liver genomic DNA by real-time quantitative PCR (qRT-PCR) and corrected by simultaneous measurement of nuclear DNA. We examined the amplification of mitochondrial cytochrome c oxidase subunit II (COII, mitochondrial-encoded gene) and β-actin (nuclear-encoded gene). The primer sequences used were as follows:

COII: 5′-TGAGCCATCCCTTCACTAGG-3′ (sense)/5′-TGAGCCGCAAATTTCAGAG-3′(anti-sense);
β-actin: 5′-CTGCTCTTTCCCAGATGAGG-3′ (sense)/5′-CCACAGCACTGTAGGGGTTT-3′ (anti-sense).

The threshold cycle (Ct) reflects the cycle number at which a fluorescence signal within a reaction crosses a threshold. In our study, the average Ct values of nuclear DNA and mtDNA were obtained for each case. mtDNA content was calculated using $\Delta Ct = \text{average } Ct_{\text{nuclear DNA}} - \text{average } Ct_{\text{mtDNA}}$ and then was obtained using the formula mtDNA content = 2 (2ΔCt).

2.6. mRNA Expression

Total liver RNA was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer′s protocol. Tissue/TRIzol® mixtures were homogenized using an Ultra Turrax homogenizer while keeping the viscosity of the solution to a minimum to ensure effective inactivation of endogenous RNAse activity. RNA samples were subjected to DNAse treatment to remove genomic DNA contamination. A total of 1 µg of total RNA was used to generate cDNA in a 20-µL reaction volume using Superscript II Reverse Transcriptase (HT Biotechnology, Cambridge, UK). PCR primers were designed using Primer Express version 2.0 (Invitrogen). We examined the mRNA expression of DNA polymerase γ (Polg), Peroxisome proliferator-activated receptor γ coactivator-1α (Pgc1α), Nuclear respiratory factor 1 (Nrf1) and Mitochondrial transcription factor A (Tfam). β-actin mRNA expression was used for normalization. Primers used were as follows:

β-actin: 5′-CTGCTCTTTCCCAGATGAGG-3′ (sense)/5′-CCACAGCACTGTAGGGGTTT-3′ (anti-sense);
Polg: 5′-GAAGAGCGTTACTCTTGGACCAG-3′ (sense)/5′-AACATTGTGCCCCACCACTAAC-3′ (anti-sense);
Pgc1α: 5′-GTCAACAGCAAAAGCCACAA-3′ (sense)/5′-GTGTGAGGAGGGTCATCGTT-3′ (anti-sense);
Nrf1: 5′-CTGATGGCCATTACATGTGG-3′ (sense)/5′-GTAAAGCCCGGAAGGTTCTT-3′ (anti-sense);
Tfam: 5′-CAACAGGGAAGAAACGGAAA-3′ (sense)/5′-GTGGCTCTGAGTTTCCGAAG-3′ (anti-sense).

An equivalent of 25 ng of total RNA was subsequently used in the amplification with 50 nmol of gene-specific primers and 4 mL of iTaq Universal SYBR Green mix (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 8 µL using standard cycle parameters on a Bio-Rad iQ5.

2.7. Preparation of Hepatic Homogenate and Isolated Mitochondria

Rat liver was gently homogenized in 10 volumes of isolation medium consisting of 220 mmol mannitol, 70 mmol sucrose, 20 mmol Tris-HCl, and 1 mmol EDTA at pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA). Aliquots of homogenate were withdrawn for further measurements while the remaining homogenate was centrifuged at 500× *g* for 10 min at 4 °C and the resulting supernatant was centrifuged at 3000× *g* 10 min at 4 °C. The mitochondrial pellet was then washed twice and solubilized in a minimal volume of RIPA buffer (50 mmol Tris-HCl (pH = 7.4), 150 mmol NaCl, 1% NP-40, 0.1% SDS, 2 mmol EDTA, 0.5% sodium deoxycholate) until addition of protease/phosphatase inhibitors and kept on ice. The mitochondrial protein concentration was determined using the Bio-Rad DC method and the mitochondrial samples were then used for Western blot analysis of catalase content.

2.8. Hepatic Lipid Peroxidation

Lipid peroxidation was determined according to Fernandes et al. [25] in liver homogenates prepared as described above, by measuring thiobarbituric acid reactive substances (TBARS), using the thiobarbituric acid assay. Aliquots of hepatic homogenates were added to 0.5 mL of ice-cold 40% trichloroacetic acid. Then, 2 mL of 0.67% of aqueous thiobarbituric acid containing 0.01% of 2,6-di-tert-butyl-p-cresol was added. The mixtures were heated at 90 °C for 15 min, then cooled in ice for 10 min, and centrifuged at 850× *g* for 10 min. The supernatant fractions were collected and lipid peroxidation was estimated spectrophotometrically at 530 nm. The amount of TBARS formed

was calculated using a molar extinction coefficient of 1.56×10^5 $mol^{-1} \cdot cm^{-1}$ and expressed as nmol TBARS·g^{-1} tissue.

2.9. Hepatic Myeloperoxidase (MPO) Activity

MPO activity was assessed in liver samples as reported by Kim et al. [26]. Briefly, tissue samples (100 mg) were homogenized in 1 mL of hexadecyltrimethylammoniumbromide (HTAB) buffer (0.5% HTAB in 50 mmol phosphate buffer, pH 6.0) and centrifuged at 13,400× *g* for 6 min at 4 °C. Then, 10 μL of supernatant were combined with 200 μL of 50 mmol phosphate buffer, pH 6.0, containing 0.167 mg·mL^{-1} 0-dianisidine hydrochloride and 1.25% hydrogen peroxide. The change in absorbance at 450 nm was measured and one unit of MPO activity was defined as that degrading 1 μmol of peroxide per minute at 25 °C.

2.10. Western Blotting

Liver tissue was homogenized in lysis buffer containing 20 mmol Tris-HCl (pH 7.5), 150 mmol NaCl, 1 mmol EDTA, 1 mmol EGTA, 2.5 mmol $Na_2H_2P_2O_7$, 1 mmol b-$CH_3H_7O_6PNa_2$, 1 mmol Na_3VO_4, 1 mmol PMSF 1 mg·mL^{-1} leupeptin, and 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) using an Ultra Turrax homogenizer and then centrifuged at 15,000× *g* in a Beckman Optima TLX Ultracentrifuge (Beckman Coulter S.P.A., Milan, Italy) for 15 min at 4 °C. The supernatants were then ultracentrifuged at 40,000× *g* in a Beckman Optima TLX ultracentrifuge for 15 min at 4 °C. The protein concentration in supernatants and cleared lysates was determined using the Bio-Rad DC method. The protein levels of POLG, PGC1α, NRF1 and TFAM were determined in the supernatants of ultracentrifuged lysates using polyclonal antibodies (Novus Biologicals, Littleton, CO, USA; Millipore, Billerica, MA, USA; Abcam, Cruz Biotechnology, Santa Cruz, CA, USA, respectively). β-actin antibody (Sigma-Aldrich) was used as control.

Catalase protein levels were measured on protein extracts from isolated liver mitochondria using a polyclonal antibody (Sigma-Aldrich) and a voltage-dependent anion channel (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as control.

2.11. Statistical Analysis

Results are expressed as means ± SEM. Statistical analyses were performed using a two-tailed, unpaired Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of Fructose-Rich Diet on Hepatic Functionality

As shown in Table 2, in fructose-fed rats, plasma levels of ALT and AST, biochemical indicators of hepatic damage, and hepatic levels of TBARS, markers of lipid peroxidation, were significantly higher compared to the controls. In addition, fructose-fed rats exhibited a significant increase in hepatic MPO activity compared to the controls. The determination of MPO activity can be used as a surrogate marker of inflammation, since it has been shown that the activity of MPO solubilized from the inflamed tissue is directly proportional to the number of neutrophils seen in histologic sections [27].

Table 2. Body weight, plasma, and hepatic parameters in rats fed a control or a fructose-rich diet.

Item	Control	Fructose
Initial body weight, g	470 ± 10	470 ± 10
Final body weight, g	540 ± 23	545 ± 15
Food intake, g·day^{-1}	32 ± 1.0	32 ± 1.0
Plasma ALT, U·L^{-1}	16.8 ± 1.0	27.3 ± 1.0 *
Plasma AST, U·L^{-1}	43.0 ± 3.1	65.2 ± 3.3 *
Hepatic lipid peroxidation, nmol TBARS·g^{-1} liver	61.5 ± 2.1	75.9 ± 2.0 *
Hepatic MPO activity, U·mg^{-1} liver	0.31 ± 0.01	0.62 ± 0.02 *

Values are the means ± SEM of nine different rats. * $p < 0.05$ compared to control diet. ALT = alanine transaminase, AST = aspartate transaminase, TBARS = thiobarbituric acid reactive substances, MPO = mieloperoxidase.

3.2. Effect of Fructose-Rich Diet on Plasma 8-OHdG Concentration

As shown in Figure 1, administration of a fructose-rich diet for eight weeks resulted in significantly increased levels of plasma 8-OHdG (+56%) compared to control rats. The reaction of intracellular ROS with DNA results in numerous forms of base damage, and 8-OHdG is one of the most abundant and most studied lesions generated. So, 8-OHdG has been used as an indicator of oxidative DNA damage in vivo and in vitro [23,28]. The above result of increased levels of plasma 8-OHdG in rats fed a fructose-rich diet suggests that such treatment induces an increase in DNA damage.

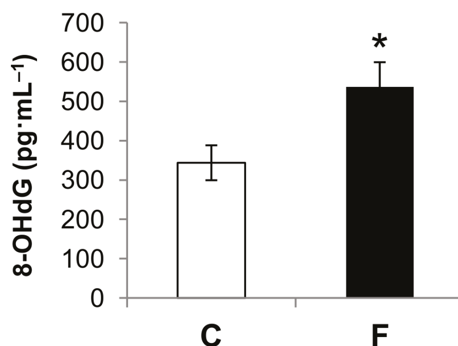

Figure 1. Effect of fructose-rich diet on plasma 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels. Values represent means ± SEM from five rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats.

3.3. Effect of Fructose-Rich Diet on Mitochondrial Catalase Expression

As shown in Figure 2, catalase protein levels in rat liver mitochondria were decreased by 35% in rats fed a fructose-rich diet compared to controls.

3.4. Effect of Fructose-Rich Diet on mtDNA Damage and Copy Number

QPCR was used to measure the levels of hepatic mtDNA oxidative damage. In rats fed a fructose-rich diet, the relative amplification of long (13.4 Kbp) mtDNA fragments was significantly reduced by 22% compared to control rats (Figure 3a). Liver mtDNA from rats fed a fructose-rich diet contained significantly more mtDNA lesions (0.24 lesion·10 Kbp^{-1}) compared to control rats (0.058 lesion·10 Kbp^{-1}) (Figure 3b).

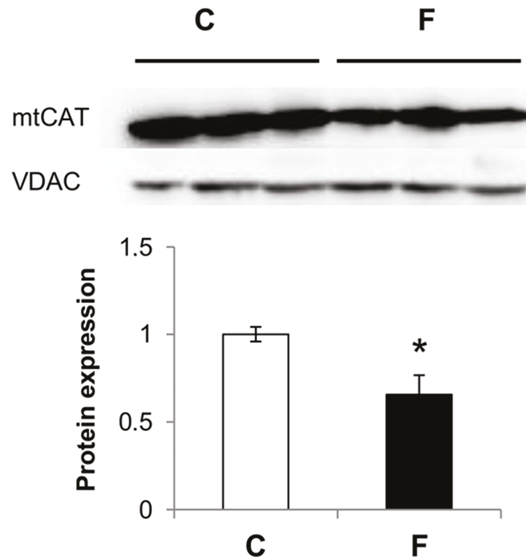

Figure 2. Effect of fructose-rich diet on catalase expression in isolated liver mitochondria. Upper panel: representative Western blot with mitochondrial catalase (mtCAT) antibody using voltage-dependent anion channel (VDAC) as an internal control. Lower panel: quantitative analysis of Western blot. Values are presented as means ± SEM from six rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats.

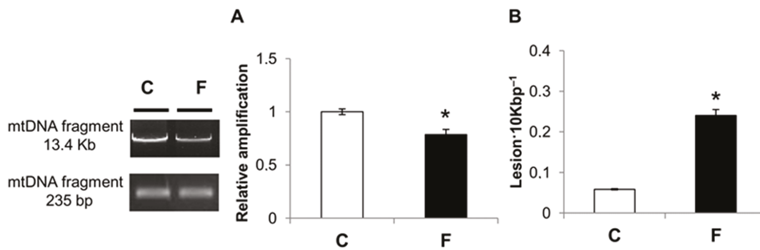

Figure 3. Effect of fructose-rich diet on mtDNA damage and lesion frequency. (**A**) mtDNA damage was evaluated in the liver by amplifying long (13.4 Kbp) and short (235 bp) mtDNA fragments by QPCR; (**B**) Frequency of mtDNA lesions per 10 Kbp per strand. Values are presented as means ± SEM from four rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats.

These results demonstrate that in rats fed a fructose-rich diet for eight weeks, there was a significant increase in oxidative damage to the mtDNA. Moreover, the mtDNA copy number was significantly reduced with the fructose-rich (−56%) versus control diet (Figure 4).

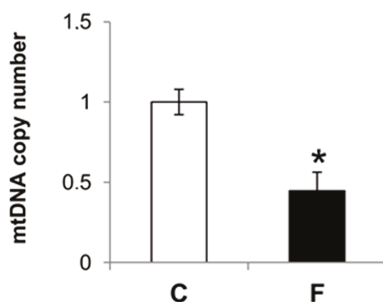

Figure 4. Effect of a fructose-rich diet on mtDNA copy number. mtDNA copy number was assessed by quantitative reverse transcription polymerase chain reaction in 10 ng of genomic liver DNA using primers for mtCOII. Expression was normalized using nuclear β-actin as an internal control. Values are presented as means ± SEM from four rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats.

3.5. *Effect of Fructose-Rich Diet on Mitochondrial POLG*

POLG is an important enzyme involved in mtDNA repair and replication. To investigate whether a fructose-rich diet caused changes in the POLG expression, qRT-PCR and Western blot analysis were performed. In rats fed a fructose-rich diet, the POLG mRNA expression (Figure 5a) and protein levels (Figure 5b) were found to be significantly decreased by 42% and 27%, respectively, compared to control rats.

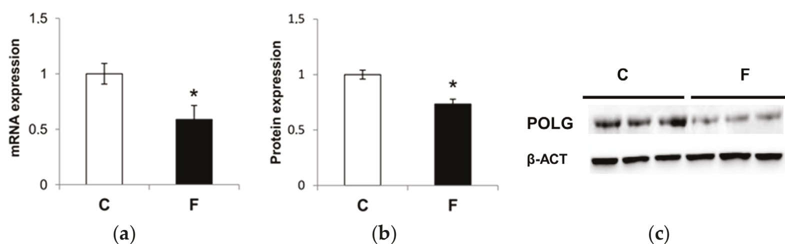

Figure 5. Effect of fructose-rich diet on DNA polymerase γ (POLG) expression. (**a**) POLG mRNA expression was measured by quantitative reverse transcription polymerase chain reaction using β-actin as an internal control. Values represent means ± SEM from four rats in each group; (**b**,**c**) Quantitative analysis and representative Western blot with POLG antibody using β-actin as an internal control. Values represent means ± SEM from nine rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats.

3.6. *Effect of Fructose-Rich Diet on Mitochondrial Biogenesis*

Hepatic mitochondrial biogenesis was evaluated by measuring the mRNA and protein levels of PGC1α, NRF1, and TFAM. PGC1α is one of the most important coactivators of mitochondrial biogenesis, which controls many aspect of oxidative metabolism, through co-activation and enhancement of the expression and activity of several transcription factors, including NRF1 [29,30]. PGC-1alpha is also indirectly involved in regulating the expression of mtDNA transcription via the increased expression of TFAM, which is coactivated by NRF1 [29,31].

In rats fed fructose-rich diets, Pgc1α, Nrf1, and Tfam (Figure 6a) mRNA expression was significantly reduced by 19%, 41% and 43%, respectively, compared to control rats. Regarding the protein levels (Figure 6b), the reductions were 27%, 29% and 27%, respectively.

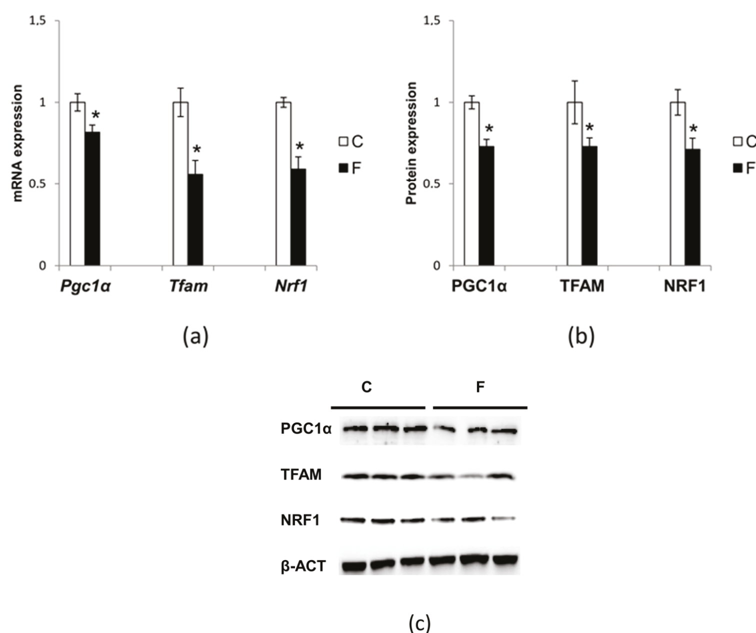

Figure 6. Effect of fructose-rich diet on peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α), nuclear respiratory factors (NRF)-1, and mitochondrial transcription factor A (TFAM) expression. (**a**) Pgc1α, Nrf1 and Tfam mRNA expression was measured by quantitative reverse transcription polymerase chain reaction using β-actin as an internal control. Values are presented as means ± SEM from four rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats; (**b**,**c**) quantitative analysis and representative Western blot with PGC1α, NRF1 and TFAM antibodies using β-ACTIN as an internal control. Values are presented as means ± SEM from six rats in each group. C: control diet; F: fructose-rich diet. * $p < 0.05$ versus C rats.

4. Discussion

In the present paper we show that long-term fructose intake is associated with increased systemic oxidative stress, as well as with marked oxidative alterations in liver cells, and in particular in hepatic mitochondria.

Further, 8-OHdG is an abundant base modification in mammalian DNA, the levels of which increase with oxidative stress [28], and it is therefore widely recognized as a biomarker of the in vivo total systemic oxidative stress [23]. Herein, we found that 8-OHdG levels were markedly increased in rats fed a fructose-rich diet (Figure 1), thus indicating the condition of increased oxidative stress at the whole-body level induced by the high intake of fructose.

In this work, we found that chronic intake of fructose is also associated with various signs of liver damage, such as increased lipid peroxidation, inflammation and cellular necrosis. Fructose intake negatively impacts liver function, since absorbed monosaccharides are firstly sent to the liver via portal blood, and up to 90% of ingested fructose is metabolized in the liver [12]. Therefore, it is conceivable that the massive flow of fructose and its handling in the liver causes a metabolic injury to the tissue.

There is accumulating evidence that fructose promotes ROS imbalance via the simultaneous enhancement of ROS production and the down-regulation of the antioxidant defense mechanisms, and the consequence is widespread damage to biological macromolecules, namely lipid peroxidation, protein oxidation, and DNA base modification. Our previous studies have demonstrated that in rats fed a fructose-rich diet for eight weeks, hepatic mitochondria showed signs of oxidative damage, both

in the lipid and in the protein component, together with the decreased activity of superoxide dismutase (SOD), one of the enzymatic components of the antioxidant defense system of mitochondria [11]. Another important member of the antioxidant system that protects mitochondria from ROS damage is mitochondrial catalase. Previous studies have identified the presence of catalase in mitochondria and its key role in oxidant defense [32–34]. In particular, these studies showed that mitochondrial catalase provides better protection than cytosolic catalase against H_2O_2-induced injury, oxidative damage, and mtDNA deletion [32–34]. Our present data of the decreased expression of catalase in rats fed a high-fructose diet (Figure 2) further support previous reports of increased oxidative stress in the hepatic mitochondrial compartment of rats fed fructose-rich diets [11]. In addition, it seems that there is a general decrease in the hepatic mitochondrial antioxidant systems induced by fructose, at variance with results obtained on fructose-fed fruit flies, where it has been shown that fructose feeding alters the activities of SOD and catalase in opposite ways [35]. Investigations on the antioxidant systems of the other cellular compartments could be useful to obtain a more complete picture.

It is well known that mtDNA is very sensitive to oxidative damage because it is located close to the inner mitochondrial membrane, where ROS are generated, and also because of the absence of protective histones and fewer repair mechanisms. Despite this, data on the effect of a diet rich in fructose on mtDNA are lacking. Since the liver is the main tissue involved in fructose processing, we evaluated the degree of oxidative damage to liver mtDNA, and the mtDNA repair mechanisms. Our results showed an increase in oxidative mtDNA lesions in the liver of rats fed a fructose-rich diet, coupled with a decrease in the mitochondrial repairing capacity, as evaluated by POLG expression, (Figures 4 and 5). Damage to mtDNA could lead to mutations during replication, possibly resulting in further important implications for cell physiology.

QPCR used in the present study was based on the principle that lesions present in the DNA block the progression of thermostable DNA polymerase on the template, resulting in decreased DNA amplification relative to undamaged DNA (amplification inversely proportional to the amount of damage). To exclude the possibility that the reduction in 13.4 Kbp mtDNA fragment amplification resulted from the loss of mtDNA molecules, we also amplified a small 235 bp mtDNA fragment. Since the probability of introducing a lesion in a small fragment is low, amplification of the 235 bp mtDNA fragment provides an accurate determination of the steady state of mtDNA levels. Then, the frequency of the mtDNA lesions was calculated using the Poisson equation.

The marked reduction in the POLG expression slows mtDNA replication and possibly affects mitochondrial biogenesis. The results reported here are in accordance with this assumption. In fact, the mtDNA copy number was significantly reduced in rats fed a fructose-rich diet (Figure 5), suggesting reduced mitochondrial biogenesis. Our finding of reduced PGC1α expression, the master regulator of mitochondrial biogenesis in rats fed a fructose-rich diet, also confirms this result (Figure 6). Further support to the reduced mitochondrial biogenesis in the liver of fructose-fed rats comes from the reduced expression of TFAM and NRF-1 found in these rats (Figure 6). In fact, TFAM is a mitochondrial transcription factor that is required to regulate the mitochondrial genome copy number [36], and NRF1 is supposed to control the key components of the protein import and assembly machinery [31], thus suggesting a broad meaning for NRF1 in orchestrating events in mitochondrial biogenesis. It is clear that a fructose-rich diet is associated with reduced mitochondrial biogenesis. The damage to hepatic mitochondrial integrity and mtDNA repair and replication mechanisms due to the fructose-rich diet could therefore lead to liver dysfunction, and consequently to metabolic diseases. Interestingly, it has been recently shown that exposure of skeletal muscle cells to fructose elicited damage similar to that found here by us in the liver, namely it increased oxidative stress and mitochondrial dysfunction, as well as decreased mitochondrial DNA content [37]. In contrast, Yamazaki et al. [38] found increased mitochondrial gene expression and mtDNA content in the liver of rats fed with fructose-containing drinking water. This different phenotype of mtDNA may be related to the severity of the metabolic syndrome and the components of the experimental design, such as the age of the rats at the start of the experiment and the duration of the treatment.

Indeed, several studies have shown that fructose consumption is associated with adverse alterations in the plasma lipid profile and severe hepatic steatosis, which is associated with necroinflammatory changes in mice [39–41]. Other studies have shown that rats fed a fructose-rich diet have increased hepatic triglyceride and cholesterol levels [42], and fructose fed to rodents at supraphysiological doses induced steatosis and steatohepatitis by de novo lipogenesis [43]. Meta-analyses have also suggested that fructose consumption is related to the risk factors for metabolic syndrome, such as increased triglyceride levels, stimulated hepatic de novo lipogenesis, and increased visceral fat [44–47].

Our current study showed that a fructose-rich diet leads to marked mtDNA damage; this diet also damaged the repair mechanism, resulting in a reduction of the mtDNA copy number and mitochondrial biogenesis. In addition, it can be speculated that mtDNA damage could lead to impairment in those systems that monitor mitochondrial health conditions and evoke appropriate stress responses, such as mitophagy, degradation of unfolded mitochondrial proteins and mitochondrial proteolysis [48].

In conclusion, our data indicate that long-term high fructose intake exerts deleterious effects on mitochondria, which may be an important factor contributing to the development of metabolic disorders, such as insulin resistance and steatohepatitis [49]. In addition, the present results could help to shed light on the recently evidenced cytotoxic effect of fructose and its metabolites in the induction of hepatocyte carcinogenesis [50].

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Author Contributions: A.L. and F.G. conceived and designed the experiments, G.L. and S.I. supervised the animal studies, A.M. and R.C. carried out all the animal studies; F.C., R.S., P.L. and A.Z. performed the experiments; F.C. and R.S. analyzed the data; S.I., F.C. and R.S. wrote the manuscript; and all the authors revised the manuscript.

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

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nutrients

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Review

High Dietary Fructose: Direct or Indirect Dangerous Factors Disturbing Tissue and Organ Functions

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Abstract: High dietary fructose is a major contributor to insulin resistance and metabolic syndrome, disturbing tissue and organ functions. Fructose is mainly absorbed into systemic circulation by glucose transporter 2 (GLUT2) and GLUT5, and metabolized in liver to produce glucose, lactate, triglyceride (TG), free fatty acid (FFA), uric acid (UA) and methylglyoxal (MG). Its extrahepatic absorption and metabolism also take place. High levels of these metabolites are the direct dangerous factors. During fructose metabolism, ATP depletion occurs and induces oxidative stress and inflammatory response, disturbing functions of local tissues and organs to overproduce inflammatory cytokine, adiponectin, leptin and endotoxin, which act as indirect dangerous factors. Fructose and its metabolites directly and/or indirectly cause oxidative stress, chronic inflammation, endothelial dysfunction, autophagy and increased intestinal permeability, and then further aggravate the metabolic syndrome with tissue and organ dysfunctions. Therefore, this review addresses fructose-induced metabolic syndrome, and the disturbance effects of direct and/or indirect dangerous factors on the functions of liver, adipose, pancreas islet, skeletal muscle, kidney, heart, brain and small intestine. It is important to find the potential correlations between direct and/or indirect risk factors and healthy problems under excess dietary fructose consumption.

Keywords: high dietary fructose; metabolites; metabolic syndrome; insulin resistance; oxidative stress; inflammation; tissue and organ dysfunction

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1. Introduction

The World Health Organization (WHO) defines metabolic syndrome (MetS) as a cluster of symptoms with impaired glucose tolerance or insulin resistance, together with two or more of the following components: raised arterial pressure, raised plasma triglyceride (TG) and/or low high-density lipoprotein (HDL) cholesterol, central obesity and microalbuminuria [1]. Central obesity and insulin resistance are acknowledged as the important causative factors in the pathogenesis of MetS [2]. MetS increases the risk of developing hypertension, cardiovascular disease (CVD), type 2 diabetes (T2DM), non-alcoholic fatty liver (NAFLD), hyperuricemia, gout and chronic kidney disease (CKD) [3].

Fructose is a monosaccharide found in fruits, vegetables and honeys. As for its sweetness, palatability and taste enhancement, fructose is widely added to processed food and beverages. High-fructose corn syrup (HFCS) is one of the most widely used food ingredients in nearly all soft drinks, canned jams, breakfast cereals and baked goods. High fructose diet and extensive commercial use of HFSC are reported to be associated with the rising prevalence of MetS worldwide [4], triggering function impairment in multiple tissues and organs. The metabolism of fructose is quite different from glucose in catabolic reaction, as well as metabolite and regulatory mechanism. Fructose is metabolized in liver via fructolysis, and the primary metabolites and by-products include glucose, lactate, free

fatty acid (FFA), very low-density lipoprotein (VLDL)-TG, uric acid (UA) and methylglyoxal (MG). Extrahepatic absorption and metabolism of fructose also take place. These metabolites are considered to be direct dangerous factors, with the potential to disturb functions of extrahepatic tissues and organs.

In addition to rapid fructolysis in liver, high fructose causes an ATP depletion that triggers inflammatory response and oxidative stress, thereby disturbing functions of local tissues and organs. Subsequently, inflammatory cytokine, adiponectin, leptin, and endotoxin are produced and become indirect dangerous factors. Fructose and its metabolites directly and/or indirectly cause insulin resistance, chronic inflammation, endotoxin secretion, autophagy and disturbance of appetite for food intake, aggravating MetS.

Therefore, we will delineate fructose-induced tissue and organ dysfunctions resulting from these direct and/or indirect dangerous factors. It will focus on the correlations between different metabolites and functional assignment of different tissues and organs in the whole body under high fructose condition.

2. Absorption and Metabolism of Fructose

Fructose is directly absorbed across the brush border of the small intestine into enterocyte by glucose transporter 5 (GLUT5) [5], and transported out of the enterocytes into systemic circulation by GLUT2, located at the basolateral pole [5]. The transported fructose is delivered into the systemic circulatory system and absorbed mainly in liver.

More than 50% of fructose is metabolized via fructolysis in liver. Fructokinase (KHK) catalyzes the first phosphorylation reaction to produce fructose-1-phosphate (fructose-1-P) and initiates fructose catabolism [6]. Aldolase B catalyzes the lysis of fructose-1-P to generate dihydroxyacetone phosphate (DHAP) and glyceraldehyde, two major components of triose-Ps. DHAP and glyceraldehyde are converted to glucose following the conventional gluconeogenesis. Part of DHAP and glyceraldehyde are converted into lactate and released into circulation, others can be reversibly metabolized to glycerol-3-phosphate (glycerol-3-P), and catalyzed by glyceraldehyde kinase. MG synthase catalyzes glycerol-3-P and/or DHAP to produce MG, which is secreted into circulation. Meanwhile, glycerol-3-P forms FFA and TG via de novo lipogenesis (DNL). Diacylglycerol (DAG), an active lipid intermediate, is produced during TG generation. Then TG is packed with apolipoprotein B100 (ApoB100), facilitating VLDL-TG production and secretion. Glyceraldehyde can also be converted to acetyl-CoA (consecutively catalyzed by glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase and pyruvate dehydrogenase), either producing FFA, or further participating in TG synthesis or entering tricarboxylic acid cycle (TCA) cycle. Another bioactive lipid intermediate, ceramide, is derived from metabolism of palmitic acid (the preliminary products of FFA synthesis). Rapid fructolysis leads to a high level of metabolic stress via ATP depletion [7], increasing AMP degradation to increase UA in liver, finally resulting in blood UA elevation. Therefore, glucose, lactate, FFA, TG, VLDL-TG, DAG, ceramide, UA and MG are overproduced and released into systemic circulation. Some of them, such as glucose, lactate, FFA, VLDL-TG, UA and MG, are delivered to extrahepatic tissues, affecting energy hemostasis or impairing tissue and organ functions. High levels of these metabolites are considered to be direct dangerous factors under high fructose condition.

Extrahepatic absorption and metabolism of fructose also take place, since GLUT5 is also widely expressed with high specificity in adipose tissue, kidney, muscle skeletal tissue, testis and brain [5]. GLUT2, a low-affinity fructose transporter, is also located significantly in kidney and small intestine [5]. It is likely that maximal physiological, postprandial concentration of fructose reaches to 1.0 mmol/L in the portal vein, and remains in the micromolar range in peripheral blood in humans and rodents [6]. Plasma fructose concentration reaches up to 1–2 mM after the intravenous fructose infusion (22 mumol$\cdot$kg$^{-1}\cdot$min^{-1}) [8], but the half-life is about 20 min in normal subjects [9]. Therefore, extrahepatic fructose uptake does not occur to a significant extent due to its very low blood level.

During the fructolysis, a high level of metabolic stress via ATP depletion is detected [7]. ATP depletion causes oxidative stress and inflammatory response to disturb the function of tissues and organs, resulting in abnormal production of insulin, inflammatory cytokine, adiponectin, leptin and endotoxin. These indirect dangerous factors are secreted into systemic circulation, further aggravating metabolic burden in tissues and organs and even perturbing appetite and food intake.

3. Direct Dangerous Factors under High Fructose Consumption

3.1. Glucose

The impact of high fructose consumption on fasting glucose level is controversial and dependent on the differences in age, energy status, and drug dose during the experiments. High fructose intake produces immediate change in hepatic and extrahepatic substrate metabolism, but the overall glucose production remains unchanged in some reports of human subjects [10] and rodents [11]. More frequently, fasting or postprandial glucose concentrations are increased after high fructose consumption in clinical trials [12] and animal experiments [13]. The elevated glucose output may cause an increase of insulin demand and trigger insulin over-release.

Increased hepatic gluconeogenesis and glucose export are considered as the major causes of systemic insulin resistance [10]. Meanwhile, fructose-induced pancreatic β-cell dysfunction causes insulin secretion as well, via activating sweet taste receptor (TR) signaling in humans and mice [14]. Impairment of β-cell mass and function inmales with high fructose diets result from dysregulation of leptin signaling and activation of protein kinase B (PKB/Akt)/Forkhead box protein (Fox) O1 in rat islets [15]. ER stress occurs in pancreatic β-cells under high fructose diet, as it is closely associated with insulin resistance, inflammation and abnormal lipid metabolism, possibly leading to glucose intolerance and insulin resistance [13].

3.2. Lactate

High fructose increases postprandial lactate level, leading to hyperlactatemia [16]. Excessive pyruvate produced by fructolysis undergoes glycolysis almost completely in liver, exporting lactate into peripheral tissues and organs. Adipose [17] and skeletal muscle [18] can also produce lactate. In adipocytes differentiated from human Simpson-Golabi-Behmel Syndrome (SGBS) preadipocytes, fructose triggers the conversion of glucose to lactate, causing lactate release [19].

Approximately 40% of the released lactate is absorbed in skeletal muscle, and then oxidized or converted to glucose. Thus, lactate elevation in blood leads to systemic insulin resistance, and can be considered an independent risk factor for the development of T2DM [20]. Lactate infusion induces insulin signaling impairment by inhibiting phosphatidylinositol 3-kinase (PI3K) and Akt activity in skeletal muscle of mice [21]. On the other hand, lactate suppresses hexokinase (HK) and phosphofructokinase (PFK) in skeletal muscle, liver, heart and kidney, resulting in glucose consumption reduction [22]. Meanwhile, glucose uptake is reduced by hyperlactatemia via suppressing GLUT4 to decrease glycolytic flux in skeletal muscle [23,24]. Glycolytic flux inhibition by high lactate even happens in the presence of insulin [25]. Mice with over-expression of GLUT4 in skeletal muscle improve insulin sensibility and glucose uptake [24]. Therefore, fructose-induced high serum lactate may mainly target skeletal muscle to reduce glucose consumption and flux, resulting in systemic insulin resistance.

Of note, a high lactate level can reduce oxygen availability and enhance inflammatory response. Hypoxia is the crucial event for glycolysis acceleration and lactate release into systemic circulation. In both humans and murine adipocytes, hypoxia inhibits insulin signaling in a hypoxia-inducible factor (HIF)-1-dependent manner by decreasing insulin receptor (IR) phosphorylation and suppressing PKB, Akt, substrate-160 kDa (AS160) and GLUT1 in response to insulin [26]. Excessive oxygen consumption correlates with the impairment of insulin-stimulated glucose uptake, which may result from the upregulation of tribbles homolog 3 (TRIB3), a negative modulator of Akt, in skeletal muscle

of rats with excess nutrients [27]. Meanwhile, interleukin (IL)-1β and IL-6 production and secretion are induced by hypoxia in adipocytes [26], which are known to trigger systemic and local insulin resistance [28]. Therefore, fructose-driven lactate overproduction is another event causing systemic and/or local insulin resistance.

3.3. Free Fatty Acids (FFAs)

High fructose consumption gives rise to hyperlipidemia [29]. Significant increase in hepatic DNL is one of the major adverse causes for metabolic burden under high fructose consumption. Therefore, increased plasma FFAs, TG and VLDL-TG levels induce hyperlipidemia, as well as TG accumulation in extrahepatic tissues and organs. Fructose-induced lipotoxicity leads to NAFLD, lipid accumulation and autophagy in skeletal muscle [30], cardiac dysfunction [31], adipose inflammation [32], CKD [33], pancreatic islet dysfunction [34], brain oxidative stress and inflammation [35].

Among the products and lipid metabolites by DNL, FFAs are the initial and primary risk factors for insulin resistance in liver and extrahepatic tissues and organs under high fructose diet. Adipose tissue, acting as a highly active metabolic and endocrine-producing organ, can also increase FFA secretion. It is reported that fructose (0.1–10 mM) directly stimulates de novo FFA synthesis in human SGBS pre-adipocytes [19]. Hepatic insulin signaling modulates glucose output to maintain serum glucose hemostasis through activating insulin receptor substrate (IRS)-1/2/PI3K/Akt pathway [11]; this pathway impairment causes hyperglycemia and compensatory hyperinsulinemia, cooperatively preceding systemic insulin resistance. Fructose feeding may upregulate hepatic carbohydrate response element binding protein (ChREBP) to activate glucose-6-phosphatase (G6Pase) and enhancing glycolytic flux, thus impairing glucose homeostasis [36].

Change of plasma FFA pattern closely links with systemic insulin resistance under high fructose diet. Reduction of plasma polyunsaturated FFAs, such as docosapentaenoic acid and docosahexaenoic acid, is closely associated with systemic insulin resistance induced by high fructose consumption [37]. Upregulation of ChREBP also activates stearoyl-coenzyme A desaturase (SCD)-1 to increase monounsaturated fatty acids (palmitoleic acid and oleic acid) and polyunsaturated fatty acids (PUFA, *n*-6 and *n*-3 polyunsaturated fatty acids) production in liver, which may account for hepatic insulin resistance in fructose-fed rats [11]. FFAs stimulate the intracellular translocation of Bcl-2-associated X protein (Bax) to the lysosome in hepatocytes, and consequently release cathepsin B, which inhibits insulin signaling by activating nuclear factor kappa B (NF)-κB to enhance tumor necrosis factor (TNF)-α secretion, leading to NAFLD [38]. Activation of a series of kinases, including protein kinase C (PKC)-θ, IκB kinase-β (IKK-β), c-jun *N*-terminal kinase (JNK) and S6-kinase may play a crucial role in insulin resistance induced by FFAs and the derived metabolites (DAG, ceramides and TG) [39]. These kinases can disturb serine phosphorylation of IRS and inhibit insulin signaling in hepatic or extrahepatic tissues and organs, such as white adipose tissue (WAT) [40] and skeletal muscle [41]. Some phosphatases also participate in fructose-induced insulin resistance. It is reported by us that fructose impairs hepatic insulin signaling by activating protein tyrosine phosphatase-1B (PTP-1B) in rats [42].

Pancreatic islet β-cell dysfunction under high fructose consumption is another adverse effect of lipotoxicity, giving rise to systemic insulin resistance. FFAs (palmitate or oleate:palmitate = 2:1) critically impair insulin secretion in isolated islets from humans [43] or C57BL/6 mice [44]. Intralipid feeding inhibits glucose-induced insulin secretion in rats, while long-term exposure of FFAs (palmitate, oleate or octanoate) further increases the ratio of proinsulin:insulin in isolated human islets exposed with fructose [45]. The dysfunction of pancreatic islet possibly results from downregulation of sterol regulatory element-binding protein (SREBP)-1c-mediated IRS-2/Akt pathway [44]. Palmitate also impairs glucose-stimulated insulin secretion and β-cell function in rat insulin-secreting INS-1 cells [46] and induces H_2O_2 formation in the peroxisomes of RINm5F insulin-producing cells [47], showing its lipotoxicity in vitro. Furthermore, palmitate exposure may induce apoptosis in isolated islets from humans [43,48], possibly through its receptor cell death-inducing DFF45-like effector b (Cideb) [48].

Oxidative stress is another negative effect under palmitate exposure, accompanied with apoptosis in βTC6 cells (a glucose-sensitive mouse β pancreatic cell line) through activating free fatty acid receptor 1 (FFAR1) [49]. Therefore, fructose-induced FFA production (especially palmitate) may disturb insulin secretion by disturbing the function of pancreatic islet β cells. High fructose consumption increases hepatic DAG level [50,51] and membrane-associated PKC activity [52,53], possibly resulting in insulin resistance. High DAG and ceramide concentrations are considered as disposal of excess FFAs in liver and cause insulin resistance. Parallel lipidomics analysis of liver tissues from mice and humans shows that DAG increase is suggested to be a hallmark of NAFLD [54]. DAG activates PKC through specific binding to PKC and promotes PKC translocation [55], suppresses Akt2 to decrease glycogen synthesis by inhibiting glycogen synthase (GS) and increases gluconeogenesis by activating G6Pase and phosphoenolpyruvate carboxykinase (PEPCK), leading to glucose release through GLUT2 in liver [56]. Fructose significantly suppresses carbohydrate utilization in mitochondria [50,51], contributing to insulin resistance in liver. PKC activation is also detected in adipose tissue, which may be associated with fructose-induced hypertriglyceridemia [52].

Fructose supplementation increases de novo ceramide biosynthesis and elevates ceramide concentrations in plasma [57], liver [58] and skeletal muscle [59], promoting local insulin resistance. Ceramide decreases the ability of insulin to activate Akt and GLUT4 translocation in 3T3-L1 adipocytes [60]. Ceramidases catalyzes ceramide to produce sphingosine, which may participate in insulin signaling impairment. The phosphorylation of sphingosine by sphingosine kinase (SphK) 1 produces sphingosine-1-phosphate (S1P). Our group shows that high fructose consumption induces SphK1/S1P signaling to activate NF-κB pathway, which accounts for lipid accumulation, insulin and leptin resistance, as well as inflammation in rat liver tissue [61]. Thus, SphK1/S1P signaling impairment is relevant to the development of MetS.

FFA overproduction mediates mitochondrial dysfunction, which may be another risk factor for insulin resistance under high fructose consumption. In different tissues of fructose-fed animals, mitochondrial dysfunction is detected, characterized by increased mitochondrial mass [62,63], decreased mitochondrial electron transport capacity [62,63], loss of mitochondrial membrane potential [64] and disturbance of antioxidant defense [32]. In turn, insulin resistance affects FFA-mediated mitochondrial uncoupling [65]. Meanwhile, decreased oxidative capacity resulting from mitochondrial impairment further suppresses FFA oxidation. Overproduction of reactive oxygen species (ROS) therefore causes insulin resistance under high fructose consumption [62,63]. Fructose consumption decreases hepatocyte NADPH oxidase 4 (NOX4) to elevate ROS production by reducing protein phosphatase 1c (PP1c) to impair insulin signaling [66]. Recently, we find that oxidative stress induces cardiac inflammation and fibrosis via scavenger receptor (CD36)-mediated toll-like receptor 4 (TLR4)/6-IL-1R-associated kinase 4/1 (IRAK4/1) signaling to suppress NOD-like receptor superfamily, pyrin domain containing 3 (NLRP3) inflammasome activation in fructose-fed rats [67]. Also, superoxide generation induced by high fructose diet increases blood pressure and blocks central insulin signaling [68].

Increased FFA uptake and ectopic deposition in extrahepatic tissues and organs, such as skeletal muscle, liver, pancreas islet and cardiovascular tissue, may result in lipotoxicity and insulin resistance under high fructose consumption. FFAs can be uptaken by several tissues via FFA transport proteins (FATPs) and CD36, both highly expressed in heart, adipose tissue, and skeletal muscle [69]. Fructose upregulates CD36 expression in adipose tissue [70] and skeletal muscle [71] to facilitate FFA uptake, resulting in local insulin resistance. Increased FFAs promote autophagy in skeletal muscle of mice with high fructose diet, likely as a compensate mechanism for clearance of lipotoxic intermediates [30].

Recently, fructose has been reported to be metabolized in several regions of brain, including cerebellum, hippocampus, cortex, and olfactory bulb, which express GLUTs and all of the enzymes in fructolysis [72], probably leading to central inflammation response. FFA elevation in plasma gives rise to hippocampal insulin signaling impairment and inflammation under high fructose consumption, since FFAs may cross the blood–brain barrier [73]. Hypothalamus is the major site

sensing energy status in the whole body. The possible mechanism relates to neuropeptides secretion via regulation of AMP-activated protein kinase (AMPK) signaling and malonyl-CoA concentration, compensating for the change in energy status [74]. Rapid fructolysis results in ATP depletion to produce more AMP. Sensing increase of the AMP/ATP ratio, AMPK is activated under high fructose consumption [75]. Moreover, peripheral indirect signals generated by fructose, including TNF-α [76] can also activate AMPK in hypothalamus. Fructose triggers AMPK/malonyl-CoA signaling in hypothalamus, subsequently increasing food intake and the risk of obesity [77]. Furthermore, fructose-induced hypothalamic AMPK activation increases hepatic gluconeogenesis by the elevation of circulating corticosterone level, further contributing to systemic insulin resistance [78].

High fructose consumption gives rise to the development of cardiovascular disease by increasing VLDL-TG, TG, cholesterol, VLDL-cholesterol and low-density lipoprotein (LDL)-c, as well as decreasing HDL in circulation [79–82]. Overproduction and secretion of VLDL-TG under high fructose consumption is proposed to be the early markers of cardiovascular metabolic diseases [79], in which hepatic DNL induction through activation of SREBP-1c plays an important role [80]. Meanwhile, elevated plasma proprotein convertase subtilisin/kexin type (PCSK) 9 induced by fructose directly influences plasma LDL-C by downregulating hepatic LDL receptor (LDLR) expression [81]. The dysregulation of PCSK9/LDLR signaling induced by fructose may cause hypercholesterolemia, possibly playing a vital role in the development of atherosclerosis [82]. Palmitic acid, the main product of FFA synthesis, is reported to increase plasma cholesterol and LDL concentrations by suppressing LDLR in liver [83], thereby increasing the risk of atherosclerosis. Increased apoC-III, one of the components of VLDL in circulation, is observed under high fructose diet [84]. Elevated apoC-III induces hypertriglyceridemia [85] and insulin resistance [86], acting as another emerging pro-atherosclerosis factor. Moreover, apoC-III production senses FFA elevation in plasma [86]. Hepatic scavenger receptor class B type I (SR-BI) acts as HDL receptor, mediating HDL transport to liver. It decreases circulating cholesterol level and has atheroprotective action [87]. Fructose feeding increases intestinal SR-BI level and basal ERK activation (downstream of MAP kinase), accounting for local tissue insulin resistance, apoB48-invloved chylomicron assembly and overproduction [88]. Therefore, under high fructose consumption, elevated FFAs and the derivate DAG and ceramide contents in circulation result in insulin resistance in liver or other tissues and organs, while increased assembly form of DNL products is responsible for cardiovascular diseases.

3.4. Uric Acid (UA)

Uncontrolled fructose catabolism in liver induces rapidly ATP depletion to overproduce UA in systemic circulation, developing hyperuricemia in humans and experimental animals [89]. Epidemiological studies reveal that hyperuricemia has close relationship with insulin resistance, inducing gout, hypertension, atherosclerosis and chronic renal diseases [90]. Elevated UA may exacerbate DNL by stimulating hepatic KHK [91] and lipogenic enzymes [92], further aggravating fatty liver. Fructose-induced serum UA elevation is responsible for ROS generation in liver [92] and extrahepatic tissues, including adipose tissue [93], skeletal muscle [94] and aorta [95]. Consequently, local oxidative stress promotes hepatic steatosis [92], skeletal oxidative stress [94], cardiac hypertrophy [96], and kidney dysfunction [97].

UA-promoted oxidative stress under high fructose consumption triggers inflammatory response, including secretion of TNF-α, IL-1β, transforming growth factor (TGF)-β1 and monocyte chemotactic protein (MCP)-1 in kidney [98,99]. Thus, renal NLRP3 inflammasome is activated under high fructose [100]. Our research group shows that activation of NF-κB signaling and NLRP3 inflammasome are driven by fructose in liver and extrahepatic tissues, causing inflammation, lipid accumulation and insulin signaling impairment in kidney and hypothalamus [101–104]. Furthermore, ROS generation under fructose-induced hyperuricemia is the crucial factor for podocyte injury by activating p38 MAPK/thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome pathway [97]. While upregulation of TLR4/myeloid differentiation primary response gene 88 (MyD88) signaling

promotes NF-κB signaling in kidney of hyperuricemic mice with high fructose diet [105]. High serum UA also causes inflammation in hypothalamic, vascular endothelium, primary gouty arthritis via acting NF-κB signaling. Pancreatic inflammation may link to elevated serum UA level induced by fructose, since in a rat insulinoma cells, the low-grade pancreatic inflammation is induced by UA [106]. Pancreas islet size and number are increased in fructose-fed rats with impaired morphology and tissue dysfunction [34]. Function disturbance of pancreas islet has potential link with hepatic inflammation. Fructose induces malondialdehyde (MDA), TNF-α and IL-6 levels in pancreas of rats, with CD68-positive cell infiltration consistently, contributing to irregular insulin secretion in pancreas [107]. These observations suggest that dietary fructose may dramatically accelerate tissue and organ inflammation by high UA-induced ROS generation.

UA-induced endothelial dysfunction is another adverse burden in hyperuricemia [108], which may promote insulin resistance and cardiovascular disease under high fructose consumption. Increase in serum UA level, correlated with hypertension and dyslipidemia, increases the risk of cardiovascular disease. Suppression of renal vasodilation may cause kidney dysfunction with increase in urine sodium retention, decrease in renal UA clearance. Elevated UA, insulin and TG in plasma are considered to be closely associated with incident hypertension. Fructose enhances the effects of a high-salt diet on blood pressure by impairing renal reabsorption of sodium in proximal tubule [109]. Hyperactivity of xanthine oxidase (XO) leads to UA production, positively being correlated with elevated serum UA and systolic blood pressure [110]. Inhibition of endothelial NO synthase (eNOS) results in UA-induced vascular insulin resistance and endothelial dysfunction under high fructose consumption [111], possibly leading to the development of hypertension. Fructose exposure activates ROS-mediated NF-κB signaling in human umbilical endothelial cells (HUVECs) [112]. As a result, tissue factor (TF) expression is elevated, which is crucial in plaque formation during atherosclerosis [113].

Hyperuricemia results from deficiency in renal UA excretion, which plays a pathogenic role in fructose-induced kidney injury. It is reported by our group that fructose induces dysregulation of renal organic ion transporters including GLUT9, renal specific transporter (RST), organic anion transporter 1 (OAT1), OAT3, and urate transporter (UAT), which cause abnormal renal UA excretion involved in hyperuricemia and renal dysfunction [114]. Meanwhile, upregulation of renal prostaglandin E_2 (PGE_2), a primary mediator of inflammation, is detected in kidney of fructose-fed rats, which may be associated with the dysregulation of renal organic ion transporters [114]. High UA creates arterial stiffness and subsequent renal dysfunction in CKD. Elevation of serum UA induced by fructose activates renin-angiotensin-aldosterone system (RAAS) in perivascular adipose tissue, increases vascular stiffness and causes inflammatory response [115]. Fructose induces chemokine overproduction, such as intercellular adhesion molecule-1(ICAM-1) [116], MCP-1 [117], IL-1β [100], TNF-α and IL-6 [118] in systemic circulation and kidney, causing the progress of CKD [117]. In kidney of fructose-fed rodents, inflammatory response has a close relationship with endothelial dysfunction characterized by high expression of iNOS, COX-2 and ICAM-1 [98,116], as well as fibrosis characterized by increased concentration of glycation end products (RAGE) and α-smooth muscle actin (α-SMA) [99].

Therefore, oxidative stress, inflammatory response and endothelial dysfunction cooperatively impair tissue functions in fructose-induced UA overproduction.

3.5. Methylglyoxal (MG)

Fructose upregulates aldolase B to increase MG production in liver, secreting into systemic circulation [119]. This high MG level blocks the allosteric bind of AMP to AMPK. AMPK is a key energy sensor to regulate carbohydrate and lipid in various tissues and organs, including liver, skeletal muscle, adipose and hypothalamus. AMPK activation inhibits acetyl CoA carboxylase (ACC) to reduce malonyl CoA, a substrate for FFA synthesis [120]. Accordingly, FFA oxidation is promoted since malonyl CoA acts as inhibitor of carnitine-palmitoyl-CoA transferase-1 (CPT1). Therefore, the impairment of AMP-sensing capacity of AMPK by MG promotes DNL, leading to the development of fatty liver and

insulin resistance under high fructose diet [121,122]. Meanwhile, AMPK suppression gives rise to gluconeogenesis and glucose output, all of which may promote fructose-induced MetS [122].

High fructose consumption induces hypertension possibly by increasing MG level in main aorta and kidney [119,123]. Elevated MG level and upregulation of aldolase B are observed in cultured rat aortic vascular smooth muscle cells [123] and aorta [124] under high fructose level, while aldolase B knockout prevents MG formation in cultured endothelial cells [125]. The promotion of vascular tone involves the upregulation of the high MG-activated renin angiotensin system. Meanwhile, MG induces advanced glycation end-product (AGE) overproduction, NF-κB activation and oxidative stress in vascular smooth muscle cells [119,123]. In fructose-fed rats, high renal MG and renin levels [126] may induce inflammation by activating receptor for AGEs and increasing NF-κB activation [123].

4. Indirect Dangerous Factors in Tissue and Organ Dysfunctions under High Fructose Consumption

4.1. Inflammatory Cytokines

Increasing evidences suggest that fructose-induced MetS is closely associated with chronic inflammation, characterized by elevated peripheral blood mononuclear cells, reduced bone marrow mononuclear cell viability [127], increased systemic inflammation cytokine concentration [127], as well as inflammation signaling activation in local tissues and organs, including liver, adipose, kidney, heart and brain [128].

Adipose tissue secrets adipokines (adiponectin, resistin, apelin and visfatin), hormones (leptin) and chemokines (MCP-1, IL-8, IL-6, IL-1, Ang-II, TNF-α, and IL-10), modulating whole energy homeostasis. Under high fructose consumption, adipose tissue is the key site, giving rise to the secretion of inflammatory cytokines in systemic circulation [129]. Adipose dysfunction can disturb energy expenditure and insulin signaling mainly through inflammatory cytokines [130]. Inflammatory response, accompanied with morphological and functional changes, increased visceral adiposity and fat accumulation, and insulin signaling impairment are detected in adipose tissue of humans or rodents with high fructose diet [29,129]. Endothelial dysfunction induced by fructose is in line with a significant infiltration of macrophages and T cells in perivascular adipose tissue [131]. Local RAAS activation partially gives rise to adipose dysfunction by promoting inflammation, insulin resistance, endothelial dysfunction and vascular stiffness [115].

Long-term treatment of IL-6, IL-1β, or TNF-α is shown to impair insulin signaling [28]. Dysfunction of adipose tissue increases plasma FFA concentration, further inducing insulin resistance [132]. Activation of Akt [11] or TLR4-mediated inflammatory signaling [133] accounts for FFA-induced insulin resistance. Autophagy is shown to be upregulated in adipose tissue of obese humans, the inhibition of which results in secretion of proinflammatory cytokines [134]. Autophagy-associated genes, including autophagy-related gene 7 (ATG7), lysosomal-associated membrane protein 2 (LAMP2) and microtubule-associated protein 1 light chain 3 beta (MAP1LC3β) are downregulated in adipose and liver under high fructose consumption [135]. TNF-α promotes ceramide and FFAs release in systemic circulation [136,137], causing insulin resistance of peripheral tissues and organs [60,138]. TNF-α induces IRS-1(Ser^{307}) phosphorylation to decrease insulin sensitivity in adipose tissue [28,139]. A series of serine kinases, including ERK, c-JNK and p38 MAPK [39] can sense lipid metabolites, and inflammatory cytokines in adipocytes and skeletal muscle cells, the activation of which under TNF-α further disturbs the functions of local tissues and organs [139]. IL-6 negatively affects insulin signaling to increase glucose uptake in skeletal muscle and suppress glucose production in liver [140]. The possible mechanism may involve the activation of serine-threonine protein kinase (LKB)1/AMPK/AS160 and JNK-mediated suppression of IRS-1 phosphorylation in skeletal muscle [140]. High fructose diet induces insulin resistance in skeletal muscle, with nuclear translocation of NF-κB 65, and subsequent secretion of IL-6, which is known to be mostly released from skeletal muscle [141]. Correspondingly, expression of inducible NOS (iNOS) and ICAM-1 is changed, suggesting that the possible role of insulin resistance in skeletal

muscle may act in an NF-κB-dependent manner [142]. Peroxisome proliferator-activated receptor (PPAR)-δ, one of the most promising pharmacological targets implicated in obesity-associated insulin resistance, is highly expressed in skeletal muscle. Fructose-induced disturbance of PPAR-δ-mediated lipid accumulation and fibroblast growth factor (FGF)-21 production, a myokine in tissue cross-talk, finally induce insulin resistance in skeletal muscle [141]. Fructose also blocks vasodilation in aorta via triggering inflammatory response. Fructose induces overproduction of NO and plasminogen activator inhibitor (PAI)-1 in endothelial cells [143]. NF-κB activation as well as TNF-α and IL-6 secretion impair insulin-triggered endothelial homeostasis partly via activating PI3K/Akt/eNOS and MAPK pathway [143]. Palmitate can induce hepatocytes to release extracellular vesicles in a death receptor 5 (DR5)-dependent manner. EVs induce mRNA expression of IL-1β and IL-6 in mouse bone marrow-derived macrophages, contributing to liver inflammation and injury [144], thereby indicating that hepatic DNL may give rise to systemic inflammatory cytokine secretion. Therefore, TNF-α, IL-1β and IL-6 secretion under high fructose consumption may account for insulin resistance, chronic inflammation and endothelial dysfunction in local tissues and organs.

Fructose consumption also induces psychological stress through inflammatory mechanism [145]. Intracellular inflammatory response is observed in brain and particularly in hypothalamus in MetS [146,147]. Hypothalamus monitors appetite, energy expenditure, carbohydrate and lipid metabolism, and blood pressure. Our group reports that inflammation response in hypothalamus causes local insulin signaling impairment in fructose-fed rats [103]. Fructose feeding induces hippocampal microglia activation through the activation of TLR4/NF-κB signaling, resulting in the reduction of neurogenesis in dentate gyrus of mice. Furthermore, fractalkine (FKN) and its receptor CX3CR1 participate in fructose-induced neuro-inflammation via the activation of TLR4/NF-κB signaling in hypothalamus [148].

Food intake is increased with central administration of fructose via affecting hypothalamic AMPK/malonyl-CoA signaling system to increase food intake in mice [77]. Moreover, fructose activates the hunger signal while depressing the satiety signal by decreasing the serum level of peptide YY3-36 (PYY) and upregulating hunger peptide neuropeptide Y (NPY) mRNA in hypothalamus, showing leptin resistance [149].

4.2. Adiponectin

Adiponectin is the most abundant adipokine secreted by adipose tissue. Dysfunction of adipose tissue leads to uncontrolled lipolysis, systemic insulin resistance, ectopic inflammation and lipid accumulation [150], aggravating the development of metabolic disorders. Fructose inhibits the secretion of adiponectin [151] and leptin [151] from adipose tissue into systemic circulation, accompanied with high plasma ghrelin concentration [152]. Adiponectin can reduce apoB and TG production to suppress VLDL release [153]. Decreased adiponectin levels in systemic circulation closely correlates with accumulation of vesical adipose and dysregulation of insulin-stimulated glucose uptake and utilization [154]. Therefore, abdominal and visceral adiposity, reduced insulin-sensitive visceral adipocytes, as well as increased body weight and fat under high fructose feeding [29,155], cause adiponectin secretion reduction [151].

Relevant work reports the crucial role of adiponectin in obesity and liver disease. Serum adiponectin concentration is decreased in rodents with increased influx of neutrophils in liver after high fructose intake, suggesting the possible modulation of neutrophil recruitment [156]. In ketohexokinase (KHK)-KO mice, fructose consumption does not change insulin sensitivity, adiponectin sensitivity and visceral obesity, indicating that the burden of MetS is closely associated with fructolysis [157]. Adiponectin receptor 1 (AdipoR1) and AdipoR2 are expressed in liver and skeletal muscle of humans, while AdipoR2 is mostly expressed in liver of rodents. Genetic variance of AdipoR1 and AdipoR2 genes is associated with liver fat contents in humans [158]. Downregulation or overexpression of AdipoR2 cause or ameliorate the development of liver fibrosis in mice [159]. Adiponectin is known for its anti-inflammatory activity. It reduces TNF-α and induces IL-10 release from Kupffer cells [160],

and upregulates chemokine interleukin 8 (CXCL8) in an AdipoR1- and NF-κB-dependent manner in primary human hepatocytes [161]. Adiponectin also blocks CD95-mediated FFA uptake [162] and FFA-induced c-JNK activation, leading to NAFLD development [163]. Adiponectin also reduces liver ROS production via activating superoxide dismutase 1 and catalase [164]. Therefore, hypoadiponection may be one of the contributors to oxidative stress, inflammation response, lipid accumulation and fibrosis in liver under high fructose consumption.

4.3. Leptin

Leptin is mainly produced in adipocytes, controlling food intake and energy expenditure. High fructose diet gives rise to leptin resistance in adipose, characterized by down-expression of leptin and leptin receptor (LEPR) in rats [135], possibly affecting autophagy [165]. Stearoyl-CoA desaturase is the rate-limiting enzyme catalyzing monounsaturated FA synthesis. Leptin prevents lipid accumulation and ameliorates insulin sensitivity in liver by downregulating stearoyl-CoA desaturase [166]. Leptin promotes liver fibrogenesis partially by inducing TGF-β1 [167].

On the other hand, high fructose consumption decreases circulating leptin concentration to increase the appetite for over-nutrients intake via affecting hypothalamus function [152]. Decreased ghrelin (major active form of ghrelin, secreted from stomach) and PYY (secreted from lower intestine) levels, increased leptin levels in serum are observed in rats with high fructose consumption [168], cooperatively increasing appetite and food intake. Leptin, ghrelin and PYY are secreted into system circulation and target hypothalamus, where is the appetite center and energy sensor of the whole body. Hypothalamus releases central appetite peptides including NPY and satiety peptide pro-opiomelanocortin (POMC) to increase food intake under fructose consumption [168]. Modulation of these appetite peptides may account for food intake reduction and be part of a defense mechanism against consumption of over nutrient diet [152].

4.4. Endotoxin

High fructose consumption-induced MetS correlates with increased intestinal permeability [169], translocation of bacterial endotoxin [170], and intestinal bacterial composition change [171], causing endotoxemia [172]. Elevation of plasma lipopolysaccharide and TNF-α levels, as well as insulin resistance in WAT of rats with high fructose diet, are restored by treatment with antibiotic or faecal samples from control donor rats [171], suggesting intestinal permeability impairment, which is closely associated with systemic or local inflammation response. KHK-C is reported to be expressed in both small bowel and cecum of mice [173]. Fructose feeding increases KHK mRNA expression in duodenum [173]. The acceleration of fructolysis in intestine may cause local inflammation and reduce tight junction protein (occludin and ZO-1) expression in intestine, documenting an increase in intestinal permeability [174]. Meanwhile, high levels of circulating inflammatory cytokines, which are often observed in fructose-fed animals or patients [127,128], may impair intestinal mucosal integrity and induce portal blood endotoxemia [175,176].

Growing evidence supports that increased intestinal permeability participates in fructose-induced MetS, giving rise to the development of NAFLD and chronic inflammation [177]. Fructose-induced endotoxemia activates Kupffer cells via upregulating TLR4/MyD88, which may be partially involved in the development of NAFLD [170], and subsequently trigger NF-κB activation and TNF-α overproduction [178]. Hepatic steatosis and inflammation are significantly ameliorated in TLR4-mutant mice compared with TLR4-WT mice [179]. Knock-out of lipopolysaccharide-binding protein (LBP) partially protects mice from fructose-induced NAFLD by blocking endotoxin from binding to TLR4 in liver [180]. Meanwhile, ROS production also participates in endotoxin-dependent development of NAFLD [181]. Gut-derived endotoxin can trigger hepatic and plasma lipocalin-2 (LCN-2) expression, as it is closely correlated with mitochondrial dysfunction and lipid peroxidation in fructose-induced NAFLD of rats [182]. Endotoxin-triggered inflammatory response in rat aorta is to induce iNOS and cyclooxygenase-2 (COX-2) [183].

5. Conclusions

Fructose is widely found in natural foods, including fruits, vegetables and honeys, and is added to commercial food additives. Overconsumption of fructose is a risk factor for the epidemic of metabolic syndrome (MetS), with dysfunctions in multiple tissues and organs including liver, adipose, pancreatic islet, skeletal muscle, kidney, heart, brain and intestine. The primary metabolites from fructolysis are produced in liver and secreted into system circulation, directly affecting tissue and organ functions; among these free fatty acids (FFA), uric acid (UA) and lactate play central roles in inducing insulin resistance in systemic and local tissue and organ, as well as causing reactive oxygen species (ROS) overproduction. These dysfunction events consequently lead to secretion of indirect dangerous factors, such as inflammatory cytokine, adiponectin, leptin and endotoxin. These indirect adverse factors give rise to inflammatory response, lipid accumulation, and endothelial dysfunction in local tissues and organs, in addition to the appetite disturbance for food intake, further aggravating the metabolic burden of fructose (summarized in Figure 1). Discussion of these direct and indirect adverse molecules in circulation helps us to uncover the clues for tissue and organ function disturbance and their correlation (Table 1). These adverse effects of high fructose consumption remind us to be cautious about excess fructose intake in our daily diet. More importantly, relevant government departments should make policies about the quality standard and safety of food additives to improve supervisions.

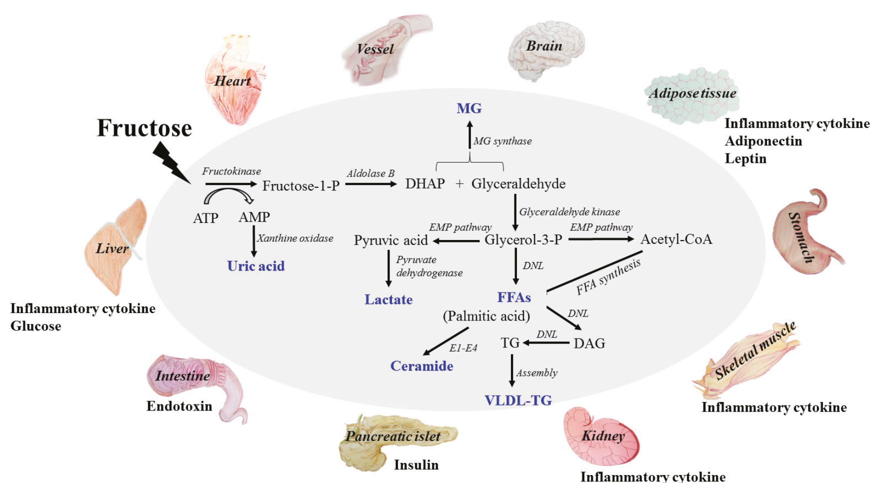

Figure 1. The metabolites of fructose catabolism and the adverse effects of high fructose consumption on tissue and organ functions in a direct and/or indirect manner. Fructose is mainly metabolized in liver to produce glucose, lactate, triglyceride, free fatty acid, uric acid and methylglyoxal. High levels of these metabolites are the direct dangerous factors. These dangerous factors impair the functions of local tissues and organs to overproduce inflammatory cytokine, adiponectin, leptin and endotoxin, which act as indirect dangerous factors. Meanwhile, glucose, insulin and ghrelin contents in system circulation are also disturbed. Fructose and its metabolites directly and/or indirectly cause oxidative stress, chronic inflammation, endothelial dysfunction, autophagy and increased intestinal permeability, and then further aggravate metabolic syndrome with tissue and organ dysfunctions. DHAP, dihydroxyacetone phosphate; TG: triglyceride; FFA: free fatty acid; UA: uric acid; MG: methylglyoxal; VLDL-TG: very low-density lipoprotein-TG. DNL: de novo lipogenesis. E1: Serine palmitoyltransferase; E2: 3-ketodihydrosphingosine reductase; E3: Ceramide synthase; E4: Dihydroceramide desaturase.

Table 1. Pathological changes of major organs and molecular mechanisms of tissue dysfunction under high fructose condition.

Organs Histopathological Changes	Dangerous Factors	Pathological Indexes		Molecular Mechanisms
		↑	↓	
Adipose tissue Inflammation response Endothelial dysfunction	FFA UA	ROS production Inflammatory cytokine flux FFA uptake Adiponectin secretion Lipid accumulation Autophagy	Insulin sensitivity Leptin sensitivity Glucose uptake Oxygen availability	PKCθ/IKK-β/c-JNK [39–41] IRS/Akt/GLUT4 [60] FATPs/CD36 [70] RAAS [115] LEPR/Stearoyl-CoA desaturase [135,165,166] ATG7/LAMP2/MAP1LC3β [135]
Brain Appetite increase Psychological stress	FFA UA MG	ROS production Inflammation cytokine flux Food intake	Insulin sensitivity Leptin sensitivity	TNF-α/AMPK/malonyl-CoA [76,77] NLRP3/NF-κB [95] TLR4/NF-κB, FKN/CX3CR1 [148] PYY, NPY [149]
Heart/vessel Hypertrophy Endothelial dysfunction Plaque formation Vascular stiffness	FFA UA	ROS production FFA uptake Vascular tone RAGE production Blood pressure	Insulin sensitivity Glucose consumption Vascular vasodilation	HK/PFK [22] FATPs/CD36 [61] CD36/TLR4/6/IRAK4/1/NLRP3 [67] AMPK/malonyl-CoA [77] XO/eNOS [110,111] PI3K/Akt/eNOS [143]
Intestine Increased intestinal permeability	UA	Endotoxin translocation Bacterial composition disturbance Dysregulation of tight junction protein	Insulin sensitivity	SR-BI/ERK/ApoB [80] KHK/Occludin and ZO-1 [173,174]
Kidney CKD Endothelial dysfunction	UA MG	ROS production Inflammatory cytokine flux Dysregulation of renal organic ion transporters NO production Urine sodium retention	Insulin sensitivity UA clearance	HK/PFK [22] XO/eNOS [110,111] NLRP3/NF-κB [92–94,96] PGE_2/Organic ion transporters [114] MAPK/TXNIP/NLRP3 [97,100–105] TLR4/MyD88/NF-κB [105]

Table 1. *Cont.*

Organs Histopathological Changes	Dangerous Factors	Pathological Indexes		Molecular Mechanisms
		↑	↓	
Liver Steatosis NAFLD Fibrogenesis Endothelial dysfunction	Lactate FFA DAG Ceramide UA MG	Gluconeogenesis Glucose export ROS production DNL Inflammatory cytokine flux Lipid accumulation Mitochondrial dysfunction VLDL-secretion	Insulin sensitivity Glucose consumption Glucose uptake Oxygen availability	IRS/PI3K/Akt, ChREBP/SCD-1 [11] HK/PFK [22,91] ChREBP/G6Pase [36] Bax/cathepsin B/NF-κB/TNF-α [38] PTP1B/IRS/PI3K/Akt [42] PKC/Akt2/GS/G6Pase/PEPCK [56] SphK1/S1P/NF-κB [61] NOX4/PTP1c [66] SREBP-1c [80] PCSK9/LDLR [82] SR-BI/ERK [88] AMPK/ACC [120] LEPR/ATG7/LAMP2/MAP1LC3β [135] AdipoR1,2/NF-κB/CXCL8 [158,161] CD95, c-JNK [161,162] TLR-4/MyD88 [170] LCN-2 [182]
Pancreatic islet Glucose intolerance Increased β-cell mass Irregular insulin secretion	Glucose FFA UA	Inflammatory cytokines flux ER stress Apoptosis	Insulin sensitivity Leptin sensitivity	TR [14] Akt/FoxO1 [15] SREBP-1c/IRS-2/Akt [44] Cideb [48] FFAR1 [49] NF-κB [106]
Skeletal muscle Inflammation response Endothelial dysfunction	Lactate FFA Ceramide UA	ROS production FFA uptake Autophagy Inflammatory cytokine flux Lipid accumulation	Insulin sensitivity Glucose uptake Oxygen availability	PI3K/Akt [21] HK/PFK [22] GLUT4 [23,24] FATPs/CD36 [23,61,62] PKCθ/IKK-β/c-JNK [40] LKB1/AMPK/AS160/IRS [140] PPAR-δ/FGF-21 [141] NF-κB/IL-6/iNOS, ICAM-1 [142]

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Abbreviations

ACC	acetyl CoA carboxylase
AdipoR	adiponectin receptor
AGEs	advanced glycation endproducts
AS160	Akt substrate-160 kDa
AMPK	AMP-activated protein kinase
ApoB100	apolipoprotein B100
ATG7	autophagy-related gene 7
Bax	Bcl-2-associated X protein
ChREBP	carbohydrate response element binding protein
Cideb	cell death-inducing DFF45-like effector b
CVD	cardiovascular disease
CPT1	carnitine-palmitoyl-CoA transferase-1
CXCL8	chemokine interleukin 8
CKD	chronic kidney diseases
COX-2	cyclooxygenase-2
DNL	de novo lipogenesis
DR5	death receptor 5
DAG	diacylglycerol
DHAP	dihydroxyacetone phosphate
ER	endoplasmic reticulum
FGF-21	fibroblast growth factor-21
FKN	fractalkine
FFA	free fatty acid
FFAR1	free fatty acid receptor 1
KHK	fructokinase
FATPs	FA transport proteins
FoxO1	Forkhead box protein O1
RAGE	glycation end products
GS	glycogen synthase
G6P	glucose-6-phosphatase
GLUT5	glucose transporter 5
HK	hexokinase
HDL	high-density lipoprotein
HFCS	high-fructose corn syrup
HUVECs	human umbilical endothelial cells
NPY	hunger peptide neuropeptide Y
HIF-1	hypoxia-inducible factor-1
iNOS	inducible NOS
IR	insulin receptor
CAM-1	intercellular adhesion molecule-1
IL	interleukin
IIRAK4/1	IL-1R-associated kinase 4/1
IKK-β	IκB kinase-β
JNK	c-jun *N*-terminal kinase
LEPR	leptin receptor

LCN-2	lipocalin-2
LBP	lipopolysaccharide-binding protein
LDL	low-density lipoprotein
LDLR	LDL receptor
LAMP2	lysosomal-associated membrane protein 2
MDA	malondialdehyde
MetS	metabolic syndrome
MG	methylglyoxal
MAP1LC3β	microtubule-associated protein 1 light chain 3 beta
MCP-1	monocyte chemotactic protein-1
NOX4	NADPH oxidase 4
NLRP3	NOD-like receptor superfamily, pyrin domain containing 3
NAFLD	non-alcoholic fatty liver
NF-κB	nuclear factor kappa B
OAT1	organic anion transporter 1
PYY	peptide YY3-36
PPAR-δ	peroxisome proliferator-activated receptor-δ
PI3K	phosphatidylinositol 3-kinase
PFK	phosphofructokinase
PAI-1	plasminogen activator inhibitor-1
PEPCK	phosphoenolpyruvate carboxykinase
PUFA	polyunsaturated fatty acid
PCSK9	proprotein convertase subtilisin/kexin type 9
PGE2	prostaglandin E2
PKB/Akt	protein kinase B
PKC	protein kinase C
PP1c	protein phosphatase 1c
PTP-1B	protein tyrosine phosphatase-1B
POMC	satiety peptide pro-opiomelanocortin
ROS	reactive oxygen species
RST	renal specific transporter
RAAS	renin-angiotensin-aldosterone system
CD36	scavenger receptor 36
SR-BI	scavenger receptor class B type I
LKB1	serine-threonine protein kinase 1
SGBS	Simpson-Golabi-Behmel syndrome
α-SMA	α-smooth muscle actin
SphK1	sphingosine kinase SphK1
S1P	sphingosine 1-phosphate
SCD-1	stearoyl-coenzyme A desaturase-1
SREBP-1c	sterol regulatory element-binding protein 1c
TR	taste receptor
TXNIP	thioredoxin-interacting protein
TF	tissue factor
TGF-β1	transforming growth factor-β1
TRIB3	tribbles homolog 3
TCA	tricarboxylic acid cycle
TG	triglyceride
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor-α
T2DM	type 2 diabetes
UAT	urate transporter
UA	uric acid
VLDL	very low-density lipoprotein
WAT	white adipose tissue
XO	xanthine oxidase

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nutrients

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Review

Role of the Enterocyte in Fructose-Induced Hypertriglyceridaemia

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Abstract: Dietary fructose has been linked to an increased post-prandial triglyceride (TG) level; which is an established independent risk factor for cardiovascular disease. Although much research has focused on the effects of fructose consumption on liver-derived very-low density lipoprotein (VLDL); emerging evidence also suggests that fructose may raise post-prandial TG levels by affecting the metabolism of enterocytes of the small intestine. Enterocytes have become well recognised for their ability to transiently store lipids following a meal and to thus control post-prandial TG levels according to the rate of chylomicron (CM) lipoprotein synthesis and secretion. The influence of fructose consumption on several aspects of enterocyte lipid metabolism are discussed; including de novo lipogenesis; apolipoprotein B48 and CM-TG production; based on the findings of animal and human isotopic tracer studies. Methodological issues affecting the interpretation of fructose studies conducted to date are highlighted; including the accurate separation of CM and VLDL. Although the available evidence to date is limited; disruption of enterocyte lipid metabolism may make a meaningful contribution to the hypertriglyceridaemia often associated with fructose consumption.

Keywords: fructose; chylomicron; very low-density lipoprotein; triglyceride-rich lipoproteins; cardiovascular disease; de novo lipogenesis; post-prandial; apoB48; gluconeogenesis; glucagon-like peptide

1. Introduction

Cardiovascular disease (CVD) remains the primary cause of death from non-communicable diseases globally, according to the World Health Organisation [1]. An elevation of fasting or post-prandial plasma triglyceride (TG) levels are both considered as independent risk factors for the development of CVD [2]. Diet is an important modifiable risk factor for CVD, including, for example, the association of sugar-sweetened beverages with increased blood pressure, plasma TG and total cholesterol in adults [3,4]. Specifically, the consumption of the monosaccharide fructose has attracted much attention, due to reports that it raises both fasting [5,6] and post-prandial TG levels [7–10]. Several issues complicate the interpretation of these findings, such as the overall energy intake and the habitual co-ingestion of fructose with glucose in the normal diet [11]. Nonetheless, dietary fructose warrants concern, particularly with regards to post-prandial hypertriglyceridaemia (HTG), as those consuming a typical 'Western' diet may spend up to 18 hours per day in the fed state [12].

A raised plasma TG concentration may result from either an increased production, or an impaired clearance, of triglyceride-rich lipoproteins (TRL), and their associated remnant particles [13]. Many dietary studies in both animals and humans have investigated the effects of fructose on the metabolism

of hepatic very-low density lipoprotein (VLDL), as a determinant of total plasma TG levels [14–16]. The liver is indeed considered to be the main organ responsible for dietary fructose metabolism [17]. However, there is also evidence that fructose-induced HTG is partly due to an increased production of intestinally-derived chylomicron (CM) particles. This has been demonstrated in a hamster model of insulin resistance [18], as well as in men with metabolic syndrome (MetS) [19].

The hydrolysis of CM and VLDL in the circulation by lipoprotein lipase (LPL) and hepatic lipase, results in the formation of smaller CM-remnant (CMR) particles, as well as VLDL remnants and low-density lipoprotein (LDL), respectively. Oxidized LDL particles have long been implicated in the pathogenesis of atherosclerosis [20]. However, both in vivo and in vitro evidence suggests that CMR also contribute to atherosclerotic plaque formation [21–23], and that this process may occur without particle oxidation [24].

Considering recent UK and US government recommendations to limit the consumption of free sugars to no more than 5% and 10% of total dietary calories respectively [25,26], this review will highlight the emerging evidence regarding the role of intestinal enterocyte metabolism in fructose-induced HTG, and thus CVD risk.

2. Fructose Absorption and Metabolism in the Enterocyte

Fructose is typically consumed either as a free monosaccharide, or as part of the disaccharide sucrose. In the UK population, adults aged 19–64 years old habitually consume a total of 95–100 g/day of non-milk extrinsic sugars, with 40–50 g/day and 15–18 g/day specifically attributed to sucrose and fructose respectively [27]. In the United States, high fructose corn syrup (HFCS), which provides glucose and fructose as free monosaccharides (55% fructose and 42% glucose; or 42% fructose and 53% glucose) [28], accounts for a significant proportion of the 54.7 g/day of fructose consumed by all ages/genders [29,30]. Although the consumption of total added sugars in the United States decreased from 100 g/day in 1999 to 77 g/day in 2008, among children and adults < 35 years old [31], a similar trend was not observed during the same period in the UK population [32].

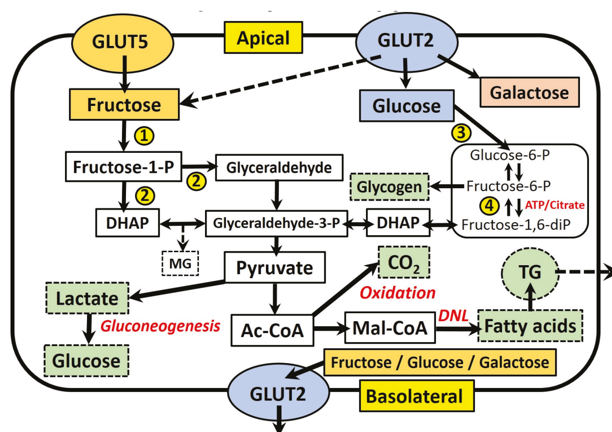

Figure 1. A schematic representation of the absorption and principal metabolic pathways of fructose and glucose within enterocytes. Fructose and glucose differ in their intracellular metabolism, with glycolytic enzymes subject to control by insulin, ATP and citrate, whereas the phosphorylation of fructose is not controlled. The main metabolic end products are glycogen (glycogenesis), CO_2 (oxidation), lactate, glucose (gluconeogenesis) and fatty acids (de novo lipogenesis). Key enzymes are numbered: (**1**) fructokinase; (**2**) aldolase B; (**3**) hexokinase/glucokinase; (**4**) phosphofructokinase. Abbreviations: Ac-CoA, acetyl-CoA; DHAP, dihydroxyacetone-phosphate; DNL, de novo lipogenesis; Mal-CoA, malonyl-CoA; MG, methylglyoxal; -P/-diP, phosphate/diphosphate; TG, triglyceride.

Figure 1 summarises the absorption and main metabolic pathways of fructose and glucose within fructose-metabolising cells, which include hepatocytes, enterocytes and kidney tubular cells [17]. Fructose is absorbed across the apical membrane of intestinal epithelial cells via an energy-independent mechanism, which requires the transmembrane transporter protein GLUT5 [33]. The expression and function of GLUT5 is upregulated in response to fructose availability, leading to increased absorption of dietary fructose [34]. This process requires the intracellular metabolism of fructose in the cytosol, via the enzyme fructokinase [35]. The majority of absorbed fructose enters the circulation across the basolateral membrane of enterocytes via the related GLUT2 transporter, which also facilitates the basolateral transport of absorbed glucose and galactose. In response to high luminal concentrations of mono- and di-saccharides, GLUT2 may also translocate to the apical membrane to increase the absorptive capacity of fructose, as well as glucose and galactose [36]. Fructose that is not directly absorbed also appears to have important interactions with the non-human cells comprising the gut microbiota. Studies in rodents indicate that the negative effects of high-fructose feeding, such as MetS and oxidative stress, are correlated with changes in the gut microbial composition, including increases in Sutterella, Coprococcus and Ruminococcus bacteria, as well as a decreased abundance of Firmicutes bacteria [37,38]. Similarly, it has also been shown that fructose-induced MetS may be ameliorated by dual probiotic treatment with ***Lactobacillus curvatus*** HY7601 and ***Lactobacillus plantarum*** KY1032 [39]. Further discussion of this expansive and novel area of investigation, however, is beyond the focus of the current review.

Within cells, fructose is rapidly converted to fructose-1-phosphate through the action of fructokinase, and subsequently to the three-carbon intermediates dihydroxyacetone phosphate (DHAP) and glyceraldehyde (GA), by the action of aldolase B. Fructose-derived carbons may then cross into the glycolysis pathway through conversion to glyceraldehyde-3-phosphate (G-3-P), making them available for energy generation through the tricarboxylic acid cycle via acetyl-CoA. Gluconeogenesis (via lactate) or glyceroneogenesis (via dihydroxyacetone phosphate) to form either glucose or glycerol, are also important. One further pathway is the conversion of acetyl-CoA, via malonyl-CoA, into new fatty acids (de novo lipogenesis, DNL). These fatty acids are esterified to form TG and either stored within intracellular lipid droplets, or packaged into lipoproteins for secretion into the circulation. The importance of DNL will be further discussed in Section 3.

Sun et al. (2012, [40]) provided one of the best available summaries to date of the quantitative disposal routes of fructose, ingested both with and without glucose or sucrose. Based on the results of 34 stable isotope tracer studies, it was concluded that oxidation (30.5%–62%), gluconeogenesis (28.9%–54%) and lactate formation (~28%) are the most important pathways, with only a minor amount of fructose channeled towards lipid formation (<1%). The authors were unable to provide an estimate of the relative contributions of glycogen (glycogenesis) and glycerol (glyceroneogenesis) formation. It was also noted that the gender, health status and intake level of subjects impacted on their conclusions.

While it is well established that the liver is the primary fructose metabolic organ, small intestine enterocytes are also known to express all of the necessary fructose-metabolising enzymes [41], as well as those associated with gluconeogenesis and lipogenesis (see Figure 1). In addition, their quantitative contribution to fructose metabolism remains unsubstantiated [17]. There have been few studies to accurately determine portal vein fructose concentrations in response to a fructose load, and to thus confirm the assumption that fructose is primarily delivered to the liver, rather than being subject to metabolism within the enterocyte. It is therefore possible that the relative contribution of the enterocyte to whole body fructose metabolism may be significant. Patel et al. (2015a) estimated that as much as 10%–30% of an absorbed fructose load may undergo catabolism within enterocytes, based on the results of studies in swine, guinea pigs and rats [42]. The same group reported portal fructose concentrations for mice consuming a diet containing 20% fructose (10% sucrose, 0% glucose) as ranging from ~0.06 mM during fasting to ~0.18 mM 8 h after feeding [43]. Unexpectedly, this diet also resulted in significantly higher portal glucose concentrations (~12.5–19 mM) than when feeding a

20% glucose (10% sucrose, 0% fructose) diet (~9–14 mM), which would appear to suggest significant gluconeogenesis of fructose within enterocytes.

The subsequent sections of this review will summarise some of the key emerging evidence supporting a significant role for the enterocyte in the observed effects of fructose consumption on HTG.

3. Fructose and Intestinal de novo Lipogenesis (DNL)

The term DNL refers to the endogenous production of newly-synthesised fatty acids (FA), which may be stored within the intracellular lipid pool or secreted within lipoprotein particles. During this process (see Figure 2), acetyl-CoA is converted into malonyl-CoA by acetyl-CoA carboxylase, which is then used as a building block by the enzyme fatty acid synthase to produce myristic acid (14:0), in turn elongated to give palmitic acid (16:0). Fatty acids may be further elongated and desaturated by other enzymes [44], although palmitic acid is considered the major product of DNL in humans [45].

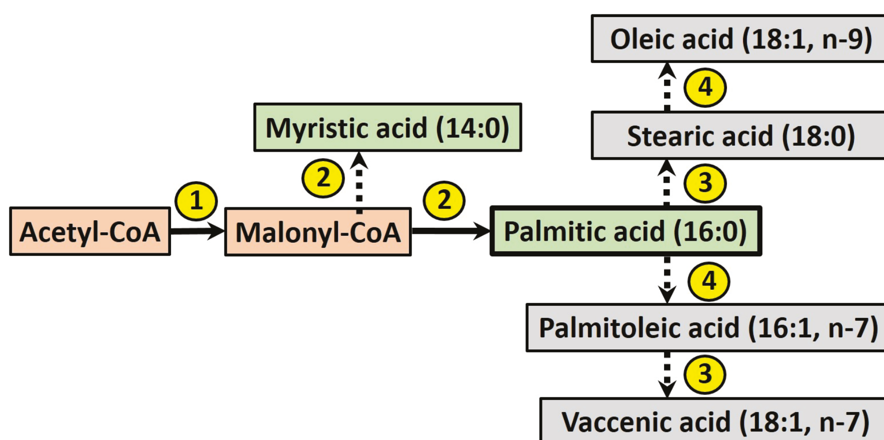

Figure 2. The process of de novo lipogenesis (DNL) in humans, where palmitic acid is the major product, which may be further elongated or desaturated to form other fatty acids. Enzymes are numbered: (**1**) acetyl-CoA carboxylase; (**2**) fatty acid synthase; (**3**) fatty acid elongases; (**4**) Δ9-desaturase.

Differences in the metabolism of fructose, as compared to glucose, may mean it is a more potent precursor for DNL. As outlined in Figure 1, fructose only requires a single phosphorylation step via fructokinase to form fructose-1-phosphate, before its conversion to the three-carbon compounds DHAP and GA. The activity of fructokinase is not regulated, whereas the metabolism of glucose is subject to insulin-mediated expression of glycolytic enzymes, as well as the inhibition of phosphofructokinase by ATP and citrate [46]. This controls the metabolism of glucose relative to cellular energy requirements. The lack of such feedback inhibition for fructose is hypothesised to provide a large precursor pool of triose-phosphates (GA, G-3-P and DHAP) for DNL, and therefore the incorporation of newly-synthesised FA into secreted lipoproteins, consequently raising plasma TG levels. The accumulation of triose-phosphates may also favour a concomitant increase in the production of methylglyoxal (MG). This acts as a precursor for the formation of advanced glycation end products (AGEs), which have gained much attention due to their implication in the pathogenesis of several age-related diseases, including type 2 diabetes and CVD [47]. Although evidence concerning the effects of fructose-derived AGEs remains limited, it has recently been posited that they may exacerbate hepatic lipogenesis via inactivation of hepatic adenosine monophosphate-activated protein kinase [48]. A thorough discussion of the effects of MG and fructose-derived AGEs is beyond the scope

of the current review, however, and readers are referred to several recent articles on the effects and potential mechanisms of AGEs [47,49–52].

Most attempts to quantify fructose-induced DNL in humans have assessed the appearance of de novo FA in VLDL secreted from the liver. For example, Schwarz et al. (2015) found that in healthy males the total hepatic DNL, as measured using sodium [1-^{13}C]-acetate, was significantly higher (7.6%) after 9 days of a 25% fructose diet versus a complex carbohydrate control diet [53]. Chong et al. (2007) utilised a [U-^{13}C]-fructose tracer to assess the amount of DNL from fructose (0.75 g/kg body weight) in 14 healthy subjects (8 male [M], 6 female [F]) and found that [^{13}C]-palmitate only accounted for ~0.4% of total circulating VLDL-TG [54]. However, it was noted that this value may have been underestimated, due to inability to account for the true isotopic tracer enrichment in the precursor pool (acetyl-CoA). A larger 2.9-fold difference in [^{13}C]-TRL palmitate was reported by Egli et al. (2013) during an oral fructose test (0.2 g/kg fat-free mass; 0.1% [U-^{13}C]-fructose) in 8 healthy males, following a 4-day diet either high (30% of energy) or low in fructose [55]. This difference did not attain significance due an unexpectedly high inter-individual variation. Nonetheless, these studies demonstrate that the ingestion of fructose is associated with some degree of de novo lipid synthesis within hepatocytes, and that fructose can be used as a precursor. There is also limited evidence, however, suggesting fructose-simulated DNL occurs in enterocytes of the small intestine.

Haidari et al. (2002) reported DNL within primary enterocytes isolated from Syrian golden hamsters fed either a high-fructose (60%) or a chow diet for 3 weeks [18]. Enterocytes from fructose-fed animals exhibited an almost 3-fold greater incorporation of the radio-isotope label [^{3}H]-acetate into FA (vs. chow-fed), indicating not only the presence of enterocyte DNL, but its upregulation in response to chronic fructose feeding. The authors also reported the incorporation of [^{14}C]-fructose into TG secreted by the enterocytes, confirming the ability of fructose to act as a DNL substrate, although the labelled TG was only approximately 25% of that secreted by hepatocytes treated in the same manner.

The capacity for intestinal DNL in humans was demonstrated by Theytaz et al. (2014) [56]. Eight healthy volunteers (4M, 4F) were given one of three mixed meals containing protein and lipid (both 0.3 g/kg body weight; ProLip), ProLip plus fructose (0.5 g/kg; Fr) or ProLip plus fructose and glucose (both 0.5 g/kg; Fr + G), with the fructose component containing 1% [U-^{13}C]-fructose to trace its metabolism. Both Fr and Fr + G meals led to detectable [^{13}C]-palmitate in the CM-TG fraction, indicating intestinal DNL with fructose as the substrate. The CM-[^{13}C]-palmitate concentration tended to be higher with Fr + G than for the Fr meal but was non-significant, although was accompanied by significantly lower fructose oxidation and gluconeogenesis, indicating the Fr + G meal favoured intestinal DNL. A similar difference was not observed for the [^{13}C]-palmitate concentration in VLDL-TG, although this was approximately three-fold greater than the CM-[^{13}C]-palmitate concentration.

Another study by the same group, utilised the same protocol (ProLip and Fr + G meals) with 8 patients who had undergone Roux-en-Y gastric bypass surgery (RYGB) and 8 control subjects. The study again demonstrated the capacity for DNL from fructose within human enterocytes [57]. In control subjects, the reported incremental area under the curve values (iAUC) for CM-[^{13}C]-palmitate were almost 2-fold greater than the VLDL-[^{13}C]-palmitate iAUC, suggesting enterocytes contributed more de novo palmitate to the overall circulating TRL-TG pool than hepatocytes. For RYGB subjects, there was no such difference between CM and VLDL. Interestingly, RYGB surgery appeared to have no effect on intestinal DNL capability, which is perhaps surprising, as this procedure typically bypasses the proximal (duodenum) small intestine and therefore prevents nutrient absorption from this section. This may indicate that DNL occurs more distally within enterocytes of the jejunum and ileum. However, such an observation is speculative.

There are several limitations to both of the aforementioned human studies, as clearly acknowledged by the authors. Perhaps most importantly, lipoproteins were separated by density gradient ultracentrifugation according to their Svedberg flotation rate (S_f), with the $S_f > 400$ fraction considered to represent predominately CM, although this may have also contained large buoyant

VLDL. Similarly, the S_f 20-400 VLDL fraction would also have contained CMR particles. Due to the low [^{13}C]-fructose content of the test meals, the [^{13}C]-enrichment in the acetyl-CoA precursor pool could not be determined, and therefore the amount of intestinal DNL from fructose could not to be accurately quantified. However, as was noted, the intestinal contribution to overall DNL may have been underestimated due to the shorter half-life, and therefore higher turnover, of CM in the circulation. Thirdly, the amount of energy differed between test meals, and there was a lack of starch and fibre. In spite of these methodological issues, these novel findings demonstrate the capacity of human small intestine enterocytes to synthesise de novo FA from fructose, which may make a more meaningful contribution to post-prandial HTG than previously appreciated.

4. Fructose Effects on Intestinal Lipoprotein Secretion

4.1. Lipid Storage and CM Secretion from the Enterocyte

The small intestine was long considered to play only a passive role with regards to TG metabolism, involved solely in the absorption, re-esterification and secretion of dietary FA as TG within CM particles, thus providing lipids to the liver, adipose tissue and muscle for either storage or energy production. However, it is now recognised that transient cytosolic lipid droplets (LD) form within enterocytes following a high-fat meal, and that these dynamic organelles are an important contributor to subsequent circulating TG levels [58,59]. In the post-prandial state, absorbed dietary TG may be directly utilized for CM formation, or stored within LD to provide TG for lipoprotein synthesis during the inter-prandial period. In this way, LD formation and mobilisation allows a gradual release of absorbed dietary lipids into the circulation, regulating the post-prandial TG response [60].

A disruption of this intestinal 'buffering' effect on post-prandial TG levels is recognized as an important contributor to the dyslipidaemia seen in MetS and type 2 diabetes in animal models and humans, whereby fasting and post-prandial CM production is increased [61].

4.2. Fructose Effects on Apolipoprotein B48 Concentration

CM formation is a complex process unique to enterocytes, which is beyond the scope of the current review and has been extensively discussed elsewhere [62–65]. One common feature is that CM contain a single structural apolipoprotein B48 (apoB48) molecule [66], which is required for their synthesis and lipidation within the lumen of the endoplasmic reticulum (ER). Therefore, plasma apoB48 concentration serves as a measure of CM particle number (including CMR). However, it must be noted that CM are known to be very heterogeneous in size, with a diameter ranging from 75 to 1200 nm depending on the TG content [62], and may be influenced by several factors, including the amount and type of dietary fat ingested [64,67], age [68], gender [69] or genetic influences [70].

Chronic feeding of a high fructose (60%) diet vs. a chow diet for 2–3 weeks has been shown to induce insulin resistance and to increase both total plasma and TRL apoB48 levels in Syrian golden hamsters [18,71], which are considered to have a similar lipoprotein metabolism to humans [72]. This was shown to be due to an enhanced apoB48 stability and secretion, with a tendency towards the production of larger TG-rich CM-like particles in the $S_f > 400$ fraction [18].

Fructose feeding may increase apoB48 concentrations by interfering with the action of insulin, which has been shown to reduce intestinal lipoprotein secretion in insulin sensitive individuals [73]. The same fructose-fed insulin resistant (FFIR) hamster model showed a lack of insulin-mediated suppression of post-prandial TRL apoB48 synthesis and secretion, in comparison to chow-fed animals, suggesting that chronic fructose may attenuate insulin signalling [74]. This was supported by a subsequent study, where sensitisation of FFIR hamsters with rosiglitazone ameliorated TRL apoB48 over-production by 50% in cultured enterocytes [75]. Further work indicated that several aspects of the insulin signaling cascade within the enterocyte may be disrupted, including decreased insulin receptor substrate 1 (IRS-1) expression and phosphorylation, as well as an enhanced basal extracellular signal-related kinase (ERK) activity, which is associated with increased activation of sterol regulatory

element binding protein (SREBP), a transcription factor known to regulate the expression of lipogenic enzymes [76]. The expression and functioning of scavenger receptor class B type I (SR-BI), a protein recognised as an important regulator of post-prandial intestinal lipoprotein production [77], is also upregulated in FFIR hamsters, and is associated with increased total apoB48 secretion [78].

Chronic fructose consumption may also perturb the action of the two gut hormones glucagon-like peptide 1 and 2 (GLP-1; GLP-2), which are co-secreted by intestinal endocrine L cells in response to nutrient availability in the lumen [79]. While GLP-1, which also stimulates insulin secretion, is known to inhibit intestinal lipoprotein production and therefore post-prandial HTG [80], GLP-2 has been shown to increase enterocyte fat absorption and lipoprotein output in response to a meal [81]. These opposing effects have been reported for Syrian hamsters, with a co-infusion of GLP-1 and GLP-2 yielding a net increase in lipid absorption, TRL-TG and TRL apoB48 levels in both healthy and FFIR animals. After chronic fructose-feeding there was a more pronounced increase in TRL-TG and TRL apoB48 concentrations, suggesting fructose may lead to either a decreased GLP-1 sensitivity or GLP-2 hypersensitivity [72]. A recent human study, however, failed to show such an effect in 65 healthy obese males (BMI 26.5–40.2 kg/m^2) following a fat-rich solid meal, with only weak correlations between GLP-1 and GLP-2 AUCs and the AUCs for both CM and total TG, as well as apoB48 [82]. It is possible that altering the meals to test the specific effect of fructose in comparison to glucose and/or a complex carbohydrate control may have revealed a relationship.

Taken together, the above Syrian golden hamster studies appear to highlight the importance of fructose-induced insulin resistance, as well as possible disruption of GLP-1 and GLP-2 signalling, as modulators of intestinal lipoprotein secretion. However, several issues such as the high-fructose content of the diets employed and the lack of comparison with either glucose or sucrose supplemented diets, make it difficult to attribute these effects specifically to fructose, as opposed to more general effects of a high sugar and/or hypercaloric diet.

Only a few studies to date have measured the effect of fructose on apoB48 concentrations in humans. Egli et al. (2013) assessed the effect of a four-day high-fructose (HFr) diet (30% energy) versus an isocaloric control diet, where fructose was substituted for complex carbohydrates, in young (21.5 $\pm$ 2.7 years), healthy (BMI 22.1 $\pm$ 1.9 kg/m^2) male subjects [55]. In comparison to the control diet, the HFr diet resulted in significantly higher plasma apoB48 and TRL-TG concentrations, both in the fasted state (approximately two-fold) and following an oral fructose challenge (0.2 g/kg), indicating an enhanced intestinal CM production.

A similar study in 9 young (21.2 $\pm$ 0.3 years) healthy weight (BMI 20.5 $\pm$ 0.8 kg/m^2) Japanese women found that the acute consumption of fructose (0.5 g/kg body weight) in combination with an oral fat tolerance test (OFTT) consisting of cream (0.35 g/kg as fat) led to a higher plasma apoB48 concentration, with a delayed peak (higher at 4 and 6 h), in comparison to either fructose, fat or an equivalent amount of glucose given alone [83]. This was also associated with significantly higher serum TG and TRL remnant-like particle (RLP)-TG. A follow-up study by the same group, again using healthy Japanese females (n = 12), varied the relative proportions of glucose and fructose (w/w) in the test meal (0.5 g/kg body weight), giving either 100% glucose or fructose (G100; F100), 90% fructose and 10% glucose (F90G10) or 55% fructose and 45% glucose (F55G45), in combination with the same amount of OFTT cream [84]. All drinks significantly increased plasma apoB48 levels at 2 and 4h post-prandially. However, 6h after all fructose-containing drinks, apoB48 levels remained significantly raised above fasted values, while that for G100 returned to pre-meal levels. Four hours after the F100 drink, apoB48 tended to be higher (5.2 $\pm$ 0.6 μg/mL) than for G100 (3.9 $\pm$ 0.6 μg/mL), although this difference did not attain statistical significance (p = 0.09), perhaps owing to the relatively small sample size, as commented on by the authors. The RLP-TG response followed the same pattern as for apoB48. Taken together, these observations may suggest either a greater CM secretion, or a delayed plasma clearance, in response to fructose.

The aforementioned study by Theytaz et al. [56] found that apoB48 iAUC values following the Fr and Fr + G meals tended to be higher than for ProLip, although this difference was not significant. It is

possible that these differences may have proved significant with a higher number of participants and thus greater statistical power [56].

Xiao and colleagues (2013) conducted one of the most rigorous investigations to date on the effects of fructose on apoB48 concentrations [85]. Seven healthy males, without impaired lipid metabolism, received an intraduodenal infusion of a 20% Intralipid (IL) solution (60 mL/h) over a 14 h period, either with or without a co-infusion of glucose (IL + G) or fructose (IL + Fr) solution (both 20%, 60 mL/h) and under conditions of an intravenous pancreatic clamp, to control for the effects of feeding on insulin, somatostatin and growth hormone. A primed constant infusion of [$^2H_3$]-leucine (10 µmol/kg bolus plus 10 µmol/kg/h for 10 h) was started 5 h into the study in order to measure apoB100 and apoB48 kinetics in the fed state. TRL-apoB48 production rate was significantly increased during both the IL + G ($p < 0.01$) and IL + Fr ($p < 0.05$) infusions (versus IL). The IL + Fr infusion also significantly raised TRL-apoB100 production rate (PR; $p < 0.05$). Although IL + G infusion led to a corresponding increase in the TRL-apoB48 fractional clearance rate (FCR; $p < 0.05$), in the IL + Fr group such an effect on FCR was not observed. These effects on apoB lipoprotein kinetics were accompanied by an increase in total plasma TG and TRL-TG in IL + G ($p < 0.05$ for both) and IL + Fr ($p < 0.01$ for both) groups.

Overall, these findings clearly demonstrate that fructose and glucose have differential effects on intestinal lipoprotein particle production and their subsequent metabolism. While TRL-apoB48 concentrations were significantly raised by both IL + G (3.06 ± 0.89 mg/L vs. 1.41 ± 0.51 mg/L saline) and IL + Fr (2.14 ± 0.50 mg/L vs. 1.39 ± 0.34 mg/L saline), with fructose this was attributed solely to an increased TRL-apoB48 PR and not due to a reduction in FCR. The reason for these differences could not be fully explained by the authors, although the lack of FCR stimulation following fructose was not due to apolipoprotein C-III inhibition of LPL, as this was not significantly affected by fructose feeding. It was suggested that glucose may chemically alter the composition of TRL particles so as to favour their metabolism and/or clearance from the circulation, as the VLDL-TG hydrolysis rate from fructose-fed, as compared to glucose-fed (10% drinking solution) rats, has been shown to be impaired, as well as the removal of VLDL-TG by the liver [86]. Whether this may explain the observed differences in apoB48 kinetics remains to be seen.

Both the animal and human studies mentioned indicate that fructose increases circulating CM concentrations, as assessed by their apoB48 content. The specific mechanisms by which fructose may differentially affect CM particle production clearly require further investigation, although a disruption of insulin and/or GLP-1 and GLP-2 signalling are potential candidates, as well as effects on the composition and thus the metabolism of CM particles by the liver and other tissues possessing LPL activity (e.g., adipose tissue).

4.3. Fructose Effects on Enterocyte CM-TG Secretion

There are very few human studies that have measured CM-TG secretion rates, or even CM-TG concentrations, because of analytical issues. As previously mentioned, the isolation of CM particles is typically achieved by density gradient ultracentrifugation, with an assumption that CM-TG corresponds to the $S_f > 400$ lipoprotein fraction. In fact, significant amounts of intestinally-derived TG have been shown to reside in the S_f 60–400 fraction (VLDL1 fraction), particularly in response to ingestion of saturated FA and during the later post-prandial phase, indicating the production of smaller, denser CM particles [87–89]. The only currently reported method by which TG of intestinal (CM) and hepatic origin (VLDL) may be definitively separated is through the use of density gradient ultracentrifugation combined with immunoaffinity chromatography. Monoclonal antibodies with specificity for apoB100 are used, which do not cross-react with apoB48, giving purified unbound (apoB48) CM and bound (apoB100) VLDL fractions [90]. Therefore, the separation of the CM-TG component within the $S_f > 60$ fraction (TRL) using immunoaffinity chromatography provides a greater specificity when assessing the enterocyte contribution to circulating TG levels. Sun et al. (2013) used this technique to develop a novel stable isotope protocol to enable CM-TG production to be measured in humans in vivo [91]. Shojaee-Moradie et al. (2013) [19] utilised the protocol to demonstrate an

increased CM-TG concentration in men with metabolic syndrome, compared to lean individuals, which was due to an enhanced CM-TG production rate, with no difference in the CM-TG clearance rate.

The aforementioned studies of Theytaz et al. (2014) [56] and Surowska et al. (2016) [57] provide some of the only available results to date regarding the effect of fructose on CM-TG levels, although they did not isolate specific CM and VLDL fractions as described above, but used the concentration of TG in the Sf > 400 fraction as a marker of CM-TG [56,57]. In the latter study, control subjects (no RYGB surgery) that consumed Fr + G in addition to ProLip tended to show an increased Sf > 400-TG response, with a larger delayed peak and higher iAUC over the 360 min time course, although these differences were not significant, perhaps owing to the low statistical power of the study and high inter-individual variation. In the first study, providing ProLip with either fructose or fructose and glucose did not affect the CM-TG response, giving equivocal iAUC values. Interestingly, although the Fr + G meal provided a higher number of calories (7.99 ± 0.14 kcal/kg body weight) than the Fr meal (5.99 ± 0.09 kcal/kg), the lack of a significant difference in total and S_f > 400-TG may suggest that fructose is a more important determinant of intestinal TG secretion than glucose, and that the effects of fructose may be irrespective of overall energy intake. However, the lack of an additional ProLip plus glucose group in this study does not allow such an observation to be confirmed. Further studies are clearly required, ideally comparing equimolar and energy-balanced glucose and fructose test meals, to determine whether fructose significantly modulates CM-TG secretion.

5. Conclusions

The importance of intestinal lipid metabolism has gained widespread appreciation in recent years, including the role of enterocyte LD and CM particle over-production as determinants of HTG, which may impact on CVD risk. Controversy persists concerning the deleterious effects of fructose consumption on human health [11,92,93], mainly due to its habitual co-ingestion with glucose in the normal diet (e.g., HFCS and sucrose) and potential confounding effects of hypercaloric and high percent fructose experimental diets. Nonetheless, human studies addressing these key issues have still reported that fructose leads to fasting and post-prandial HTG [5,9,10,55]. Equally, it has recently been shown that restricting the fructose content of the diet (isocaloric substitution with starch) has beneficial effects in children with obesity and MetS, including reductions in fasted TG, total apoB, apoC-III and LDL cholesterol levels, as well as an increase in LDL particle size [94,95].

There are several putative mechanisms by which fructose consumption may contribute to HTG through disruption of enterocyte TG metabolism. Emerging evidence from rodents and humans indicates that enterocytes may make a more meaningful contribution to fructose metabolism than previously thought [42,57]. This appears to include disposal of fructose carbons via DNL, providing de novo FA to the circulation. Specific consumption of fructose may also enhance CM secretion, acting through an attenuation of the endogenous control of lipoprotein secretion by insulin, GLP-1 and GLP-2. Although evidence supporting these effects is at present in its infancy, future investigation may reveal dysregulation of intestinal lipid metabolism as an important factor in the observed effects of fructose on post-prandial TG.

Ideally, future studies should compare the long-term effects of modest, biologically relevant amounts of fructose, as typically consumed in the habitual diet, in combination with sucrose, glucose and/or starch, and as part of an isocaloric weight-maintenance diet. It would also be useful to vary the relative proportions of fructose and other sugars administered, as together this would address the confounding issue of differential fructose metabolic handling in the presence/absence of other simple and complex carbohydrate sources (e.g., the influence of co-ingestion with glucose). Studies should also be conducted in both healthy individuals as well as in those at an increased risk of CVD, such as subjects with MetS and type 2 diabetes. Where possible, quantitative stable isotope methodology should be employed to specifically trace the metabolism of fructose, as compared to other sugars. Also, the accurate separation of CM and VLDL within isolated TRL fractions based on their specific apoB content, would enable confirmation of the relative contribution of enterocytes to fructose-induced

HTG. If this is found to be significant, it may provide further evidence to support the recent UK (5%) and US (10%) public health recommendations to restrict the amount of dietary calories obtained from free sugars [25,26] for cardiometabolic disease prevention.

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Review

Inborn Errors of Fructose Metabolism. What Can We Learn from Them?

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Abstract: Fructose is one of the main sweetening agents in the human diet and its ingestion is increasing globally. Dietary sugar has particular effects on those whose capacity to metabolize fructose is limited. If intolerance to carbohydrates is a frequent finding in children, inborn errors of carbohydrate metabolism are rare conditions. Three inborn errors are known in the pathway of fructose metabolism; (1) essential or benign fructosuria due to fructokinase deficiency; (2) hereditary fructose intolerance; and (3) fructose-1,6-bisphosphatase deficiency. In this review the focus is set on the description of the clinical symptoms and biochemical anomalies in the three inborn errors of metabolism. The potential toxic effects of fructose in healthy humans also are discussed. Studies conducted in patients with inborn errors of fructose metabolism helped to understand fructose metabolism and its potential toxicity in healthy human. Influence of fructose on the glycolytic pathway and on purine catabolism is the cause of hypoglycemia, lactic acidosis and hyperuricemia. The discovery that fructose-mediated generation of uric acid may have a causal role in diabetes and obesity provided new understandings into pathogenesis for these frequent diseases.

Keywords: fructose; inborn errors of metabolism; toxicity

1. Introduction

People in developed countries may ingest up to 50 to 100 g fructose equivalents daily in their diet and the use of this sugar in foods and drinks is increasing globally [1,2]. Fructose is almost exclusively derived from the diet. It is found in its free form in honey, fruits, and many vegetables, and is associated with glucose in the disaccharide sucrose in numerous foods and beverages. Sorbitol, widely distributed in fruits and vegetables, is converted to fructose in the liver by sorbitol dehydrogenase. Recently it has been demonstrated that fructose is also produced endogenously from glucose by the human brain via the polyol pathway [3]. Exogenous fructose is absorbed through glucose transport proteins (GLUT) 5 and 2 across the intestinal epithelium and is metabolized (mainly in the liver) by the enzymes fructokinase, aldolase B, and triokinase. The majority of the fructose is converted into glucose, which can be either stored as glycogen or released as plasma glucose. Part of the fructose is oxidized or converted into lactate and fatty acids through the process of de novo lipogenesis [4]. Dietary sugar has particular effects on those whose capacity to metabolize fructose is limited. Three inborn errors are known in the pathway of fructose metabolism; (1) essential or benign fructosuria due to fructokinase deficiency; (2) hereditary fructose intolerance (HFI); and (3) fructose-1,6-bisphosphatase (FBPase) deficiency. The description of the clinical symptoms and biochemical anomalies in the three inborn errors of metabolism (IEM) is preceded by an outline of the metabolism of fructose [5]. The potential toxic effects of fructose in healthy humans also are discussed: it is essential to understand these toxic effects in order to comprehend the pathophysiology of HFI and of FBPase deficiency.

2. An Overview of Fructose Metabolism

2.1. Enzyme of Fructose Metabolism

Fructose utilization in humans and animals occurs mainly in the liver, kidney, and small intestine [6]. Unlike glucose, fructose can enter muscle cells and adipocytes in the absence of insulin by using facilitative GLUT. However, glucose can enter muscle and adipose tissue in the absence of insulin albeit in very small quantities. After apical transport mediated by GLUT5, fructose is transported across the basolateral membrane by GLUT2 [7]. The predominance of liver, kidney, and small intestine in fructose metabolism is based on the presence of the three enzymes—fructokinase, aldolase type B, and triokinase—which convert fructose into intermediates of the glycolytic–gluconeogenic pathway (Figure 1) [8]. Both fructose and glucose can be degraded into triose-phosphate and lactate and, thus, yield glycolytic intermediates. Their two initial metabolic steps, however, differ: fructose at physiological concentration is not readily phosphorylated by hexokinase, the enzyme catalyzing the synthesis of glucose-6-phosphate from glucose in all cells of the organism; instead, it is first phosphorylated to fructose-1-phosphate (F-1-P) by a specific enzyme, fructokinase, then converted into triose-phosphate by a second enzyme, aldolase B [9]. These apparently minor metabolic variations, however, have profound metabolic consequences, as discussed further.

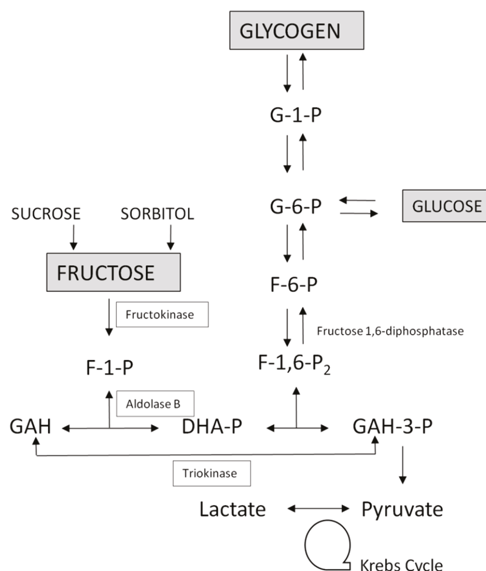

Figure 1. Fructose metabolism. Fructose enters the cell via the fructose transporter GLUT5 and GLUT2. Liver enzyme fructokinase phosphorylates fructose to fructose-1-phosphate. This is cleaved by fructose-1-phosphate aldolase (aldolase B) to form dihydroxyacetone phosphate and glyceraldehyde. Glyceraldehyde is then phosphorylated by triokinase to glyceraldehyde-3-phosphate. Thus, the intermediary metabolites of fructose enter glycolysis and the Krebs cycle as triose phosphates. Adapted from [10] and [11]. F: fructose; G: glucose; P: phosphate; DHA: dihydroxyacetone; GAH: glyceraldehyde.

2.2. Fructose Toxicity

After the discovery of HFI, fructose toxicity was thought to be limited to individuals with the aldolase B defect. In the late 1960s, deleterious effects of high dose intravenous (IV) fructose were also recognized in healthy persons. Hyperuricemia [12] and lactic acidosis [13] were the

prominent findings and are due to the influence of fructose on purine catabolism and glycolytic pathway, respectively. These observations have led to the recommendation of great caution when using fructose in parenteral nutrition [14,15]. IV fructose bypasses the regulatory steps that control glucose catabolism in the following ways: (1) entry of fructose into cells is insulin-independent; (2) IV feeding with large quantities of fructose depletes cellular inorganic phosphate and lowers the concentration of ATP; and (3) in liver, fructose evades the rate-limiting control mechanism by entering glycolysis as dihydroxyacetone phosphate or glyceraldhehyde 3-phosphate [10]. Resulting accumulation of F-1-P provokes important changes in the concentration of several other metabolites (e.g., glucose, lactate, and uric acid) which explains the toxic effects of fructose. In hypoxic conditions, such as shock or severe trauma, IV fructose may cause a massive unregulated flux of metabolites through glycolysis and fatal lactic acidosis [16]. Fructose toxicity is not only limited to the IV route as, orally, fructose consumption can also be deleterious when consumed in large quantities in the daily diet. In recent years, increased consumption of fructose, particularly from sweetened beverages, has been associated with an increased prevalence of obesity, metabolic syndrome, type 2 diabetes, and gout [4,17]. Compared to other nutrients, such as glucose or fat, fructose is first processed in the splanchnic organs and then released as glucose, lactate or VLDL-TG into the systemic circulation. These products, in turn, promote intrahepatic fat deposition, hepatic insulin-resistance, hypertriglyceridemia, hyperuricemia, and hypertension. These fructose-induced effects were proposed as being markers of cardiometabolic diseases. Nevertheless some controversies exist on how, and to what extent, those changes alter cardiovascular risk [18].

3. Inborn Errors of Fructose Metabolism: A Model for Fructose Toxicity

Inborn errors of fructose metabolism are summarized in Table 1.

Table 1. Summary of enzyme defect, main clinical symptoms, and treatment of inborn errors of fructose metabolism.

Name of the Disease	Enzyme Defect	OMIM Number	Main Clinical Symptoms	Gene Mutations/ Inheritance	Treatment
Essential fructosuria	Fructokinase	229800	Asymptomatic	*KHK*/AR	• No treatment
Hereditary fructose intolerance	Aldolase B	229600	Abdominal pain, nausea, hypoglycemia symptoms, shock-like syndrome after fructose ingestion	*ALDOB*/AR	• Withdrawal of all sources of fructose (food, drugs, liquids, parenteral infusions) • Intravenous glucose for hypoglycemia • Supplementation with folate and vitamin C
FBPase deficiency	FBPase	229700	Life-threatening episodes of hypoglycemia, coma triggered by a febrile episode, fasting or large amount of fructose ingestion (~1 g/kg BW)	*FBP1*/AR	• Avoiding catabolic triggers • Frequent feeding (use of slowly absorbed cornstarch) but to avoid overfeeding • Hypoglycemia correction with oral +/− IV glucose • In the absence of triggers no CH supplements needed • Restriction of fructose, sucrose and sorbitol

AR: autosomal recessive, BW: body weight, CH: carbohydrate, FBPase: fructose-1,6-bisphosphatase.

3.1. Essential Fructosuria-Hepatic Fructokinase Deficiency (OMIM 229800)

This rare and benign error of metabolism was first described in 1876. The disorder is caused by the inherited deficiency of fructokinase. Since the disorder is asymptomatic and harmless, many cases may remain undetected and the detected ones unpublished. Prevalence is, therefore, unknown. Ingested fructose is partly excreted unchanged in the urine and the rest is metabolised by an alternative pathway, namely, conversion to fructose-6-phosphate by hexokinase in adipose tissue and muscle [19]. Affected persons are usually discovered on routine urinalysis by the presence of reducing sugars with a negative reaction with glucose oxidase. The misdiagnosis of diabetes is avoided only when the non-glucose nature of the sugar is recognized. After an oral or IV load of fructose (1 g/kg body weight), blood fructose increases rapidly, far the level seen in controls and declines slowly. Between 10% and 20% of the administered dose is excreted in the urine as compared with 1%–2% in normal subjects [20]. The loss of fructose into the urine in this condition illustrates well the fact that fructose, having escaped hepatic metabolism, is poorly metabolized in extrahepatic tissues. Using ^{31}P magnetic resonance spectroscopy (MRS) to measure changes in liver metabolite concentrations in adults with fructosuria, Boesiger et al. found that concentrations of F-1-P, ATP, and inorganic phosphate remained unchanged, confirming that fructokinase was indeed inactive [21]. Heterozygotes appear to excrete no more fructose after an oral load than normal subjects [22]. An oral load with 50 g of fructose produces a further increase of fructose-3-phosphate in the red cells. Erythrocytes were shown to metabolize fructose to fructose-3-phophate. Concentrations of fructose-3-phophate were 3 to 15 times higher in adult with essential fructosuria than in healthy controls [23]. Accumulation of fructose-3-phosphate in the lens of diabetic rats raised the hypothesis of a diabetogenic effect of fructose-3-phophate [24]. Nevertheless, neither cataracts nor diabetes have been reported in subjects with essential fructosuria and is likely of a benign nature.

3.2. Hereditary Fructose Intolerance and Accumulation of F-1-P (OMIM 229600)

HFI is caused by a deficiency of the second enzyme of the fructose pathway, aldolase B which splits F-1-P into dihydroxyacetone phosphate and glyceraldehyde in the liver, small intestine and proximal renal tubule (Figure 1). The triose products of aldolase B are key intermediates in glycolysis and gluconeogenesis (GNG), but many of the manifestations of HFI are attributable to the toxicity of non-degraded F-1-P. Because of the high activity of fructokinase, intake of fructose results in accumulation of F-1-P and the trapping of phosphate. This has two major effects: (1) Inhibition of glucose production by blockage of GNG and of glycogenolysis which induce a rapid drop in blood glucose and (2) overutilization and diminished regeneration of ATP. This depletion of ATP results in increased production of uric acid and a release of magnesium as well as impaired protein synthesis and ultrastructural lesions which are responsible for hepatic and renal dysfunction. The toxic effects of fructose in HFI can be fatal. Patients with HFI only develop symptoms when they are exposed to fructose either as the monosaccharide, or in sucrose or sorbitol. Vomiting, diarrhea, abdominal pain, hypoglycemia, hepatomegaly, jaundice, and renal failure can ensue. IV administration of fructose to healthy subjects also induces the metabolic derangements described above (including the drop in ATP and inorganic phosphate and the rise of urate and magnesium) to an equivalent extent, although they are more transient than in patients with HFI, as demonstrated by ^{31}P-MRS [21]. The similarity between HFI and IV fructose in healthy persons is striking, indicating the importance of upstream control on fructose rate entry in the cells which is dependent on blood fructose concentration. This is even more underscored when information about essential fructosuria is added, indicating that unmetabolized fructose is innocuous, while fast partial metabolism in the liver seems to be the genesis of fructose toxicity. Diagnosis of HFI is suspected from a detailed nutritional history and the clinical picture. As soon as HFI is suspected, all sucrose, fructose, and sorbitol must be eliminated from the diet and medications. It should be remembered that sucrose and sorbitol, often used as excipients in pediatric formulations, are not always harmless and may trigger metabolic decompensation in patients not yet diagnosed with HFI [25]. Prognosis is excellent and recovery within a few days after fructose eviction.

Diagnosis is confirmed by molecular analysis of the *ALDOB* gene. If no mutation can be found despite a strong clinical and nutritional history suggestive of HFI, demonstration of deficient (<10%) aldolase activity in liver sample will confirm the diagnosis. In the past IV fructose tolerance test has served as a functional method of diagnosing HFI [26]. However due to the risk of severe and eventually fatal adverse metabolic effects induced by fructose infusion and the lack of availability of D-fructose for IV use, this test is no longer carried out.

Few studies have examined the effect of fructose ingestion in heterozygotes subject for HFI. HFI prevalence in central Europe is estimated to be 1:26,100 [27]. Based on this, a carrier frequency is predicted between 1:55 and 1:120 [28]. Although heterozygous carriers have no reported metabolic defects, they may have enhanced uric acid responses to IV and oral fructose load (50 g) according to some reports [26,29]. Assuming that estimated oral fructose consumption might reach 50 g/day in the United States, heterozygous carriers may be predisposed to gout [4]. Speculation about fructose intake and gout was raised about 50 years ago [12], and in some patients with gout hyperuricemia, the frequency of gouty attack was reduced by a fructose restricted diet [30]. It seems possible that those who benefited were heterozygous for HFI. Recently a meta-analysis of prospective cohorts studies investigating total fructose consumption and its association with incident hyperuricemia and gout concluded with a significant overall association. However the strength of evidence for the association between fructose consumption and risk of gout was low, meaning that further prospective studies are needed to conclude on which extent fructose may mediate the risk of hyperuricemia and gout [31,32].

There is evidence from mouse model that high amount of fructose consumption can also lead to non-alcoholic fatty liver disease (NAFLD) in the liver and obesity [33]. The recent generation of a mouse model ($Aldo2^{-/-}$) clearly phenocopies the human condition of HFI, which might provide a valuable resource for answering remaining questions about fructose metabolism and liver damage [34].

3.3. Fructose-1,6-Bisphosphatase Deficiency (OMIM 229700)

FBPase deficiency is an autosomal recessive disorder and impairs liver formation of glucose from all gluconeogenic precursors, including dietary fructose. Its frequency is much lower than HFI and estimated to be around 1–9/100,000 [35]. Less than 100 patients are described and very few affected adults have been reported. FBPase is a key enzyme of GNG. Its inactivity prevents the endogenous formation of glucose from the precursor lactate, glycerol, and gluconeogenic amino acids, such as alanine. Infants become symptomatic when they are dependent on GNG within the first weeks of life [36]. During fasting, maintenance of blood glucose depends on glycogenolysis, and the duration of normoglycemia thus depends on the amount of available liver glycogen. When hypoglycemia is reached, the blood glucose level is unresponsive to injected glucagon and the defect provokes an accumulation of gluconeogenic substrates (lactate, alanine, and glycerol) [37]. Investigation of fasting GNG in a patient with liver-and kidney specific FBPase deficiency, using an in vivo stable isotopes dilution method, showed that GNG contributed to up to 20% of whole body glucose endogenous production, raising the hypothesis of a specific FBPase isoform activity in muscles. This observation contributed to the knowledge of the potential role of extrahepatic and extrarenal tissue in glucose homeostasis during fasting [38]. Tolerance to fructose in FBPase deficiency is higher than in patients with HFI. ^{31}P-MRS of the liver following IV fructose (200 mg/kg BW) administration documented a slower decrease in the F-1-P accumulation and a delayed recovery of the ensuing depletion of Pi and ATP in adult patients with FBPase deficiency relative to healthy controls [21]. Although in FBPase deficiency, fructose changes of metabolite is less pronounced than in HFI, fructose tolerance test are not without risk and severe neurological complications has been reported following the administration of fructose or glycerol [39,40]).

Childhood manifestations of FBPase deficiency include hypoglycemia and lactic acidosis. Episodes are triggered by catabolic triggers, such as fever, diarrhea, and prolonged fasting. Metabolic disturbances seem to diminish with increasing age and adult patients are more tolerant of catabolic stressors, as well as sugar intake (sorbitol, fructose, or glycerol ingestion), with the exception of

pregnancy, which is a serious risk factor for metabolic decompensation, due to its increased glucose requirements. Diagnosis of FBPase deficiency is established by molecular analysis of the *FBP1* gene and eventually FBPase activity in liver samples if no mutation is found [41]. Prognosis is excellent as long as metabolic decompensation is prevented by avoidance of prolonged fasting, as well as fructose and sucrose restriction in the diet. Hypoglycemia should be treated properly with the administration of exogenous glucose or by glucose infusion. Pre-pregnancy education and self-monitoring of blood glucose to prevent hypoglycemia, as well as continuous glucose infusion during fasting delivery, reduce maternal and fetal complications [42,43].

Sometimes rare diseases serve as a model for developing therapeutic strategies for more common diseases; in the case of FBPase, being a gluconeogenic enzyme, the role of selective inhibitors of FBPase on glucose control has been raised as a potential drug therapy for type 2 diabetes [44]. In animal models of type 2 diabetes, inhibition of FBPase effectively lowers endogenous glucose formation without causing hypoglycemia [45]. Preliminary results in 42 patients with type 2 diabetes demonstrated a modest glucose-lowering effect [46]. Some phase 2 clinical studies are in progress [47]. Safety concerns about hypoglycemia and lactic acidosis may limit the clinical usefulness of FBPase inhibitors.

4. Conclusions

Studies conducted in patients with inborn errors of fructose metabolism helped to understand fructose metabolism and its potential toxicity. Influence of fructose on the glycolytic pathway and on purine catabolism is the cause of hypoglycemia, lactic acidosis, and hyperuricemia under certain conditions and in certain populations. Toxicity was first thought to be limited to individuals with inborn errors of fructose metabolism, however, a few decades thereafter, deleterious effects of fructose were also recognized in healthy persons when exposed to large quantities of IV or oral fructose. Following those studies, there has long been interest in the metabolic effect of dietary fructose. The discovery that fructose-mediated generation of uric acid may have a causal role in diabetes and obesity provided new understandings into pathogenesis for these frequent diseases. Nevertheless, the contribution of those effects on cardiovascular risk remains unclear and many mechanisms still need to be clarified before drawing definitive conclusions. Future IEM research can yield important insight into more common conditions. Additionally, rare diseases are often more extreme and have a more straightforward etiology than their common counterparts and, therefore, provide models of disease that are easier to study. Rare diseases are fundamental to understanding common diseases.

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Article

Associations of Dietary Glucose, Fructose, and Sucrose with β-Cell Function, Insulin Sensitivity, and Type 2 Diabetes in the Maastricht Study

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Abstract: The associations of glucose, fructose, and sucrose intake with type 2 diabetes mellitus (T2DM) have been inconsistent. Furthermore, there is a lack of studies focusing on early markers of T2DM that provide insight into the process of T2DM progression: impaired pancreatic β-cell function (BCF) and insulin sensitivity. This study evaluated associations cross-sectionally in a population-based cohort consisting of 2818 individuals (mean ± SD age 59.7 ± 8.18, 49.5% male, n = 120 newly diagnosed T2DM). Glucose, fructose, and sucrose intake were assessed by a food frequency questionnaire. Glucose metabolism status, insulin sensitivity, and BCF were measured by a seven-points oral glucose tolerance test. Linear regression analysis revealed a positive association of glucose intake with insulin sensitivity in the fully adjusted model (standardized beta (95% CI) 0.07 (0.05, 0.14) SD for ≥23 g vs. <10 g of glucose). Fructose and sucrose intake were not associated with insulin sensitivity after full adjustments. In addition, no associations of dietary glucose, fructose, and sucrose with BCF were detected. In conclusion, higher intake of glucose, not fructose and sucrose, was associated with higher insulin sensitivity, independent of dietary fibre. No convincing evidence was found for associations of dietary glucose, fructose, and sucrose with BCF in this middle-aged population.

Keywords: beta-cell function; diet; fructose; glucose; insulin sensitivity; prediabetes; sucrose; type 2 diabetes

1. Introduction

Lifestyle modifications, including the adoption of a healthier diet, have been suggested to reduce type 2 diabetes mellitus (T2DM) risk by 40%–70% [1]. Previous studies on the association of dietary intake of total mono- and disaccharides with T2DM have shown conflicting results. These conflicting results might partly be explained by opposite associations of individual mono- and disaccharides with T2DM [2]. The most commonly consumed, and most frequently studied individual mono- and disaccharides are glucose, fructose, and sucrose [3]. In some studies, positive associations of glucose and fructose with prediabetes [4] and T2DM [2,5] have been observed, whereas others revealed inverse [6] or absent associations [7]. Regarding sucrose intake, some studies showed inverse associations with prediabetes [8] or T2DM [2,9], whereas others showed nonsignificant associations [5,6,10].

β-cell function (BCF) and insulin sensitivity deteriorate years before T2DM diagnosis [11]. To intervene early in the process of T2DM development and to increase insight in the putative effects of mono- and disaccharides on more detailed level of defects in glucose metabolism, it is crucial to study determinants of BCF and insulin sensitivity. Defects in the intracellular insulin signaling pathway result in decreased glucose uptake by insulin-sensitive tissues [12]. If insulin sensitivity decreases, pancreatic β-cells normally upregulate insulin secretion in order to maintain normal blood glucose levels [13,14]. However, when β-cell function is impaired, plasma glucose levels rise into the prediabetic and diabetic range [13,14].

Studies evaluating the effects of mono- and disaccharide intake on insulin sensitivity are scarce and have resulted in conflicting findings [15–17]. Furthermore, to the knowledge of this study's authors, no earlier studies focused on non-fasting measures of insulin sensitivity in order to provide important insights into overall insulin resistance (hepatic and peripheral insulin resistance), while fasting indices reflect mainly hepatic insulin resistance [18]. The effects of mono- and disaccharide intake on BCF are unknown. Therefore, the aim of the current study was to investigate the individual associations of glucose, fructose, and sucrose intakes with BCF and insulin sensitivity as primary outcomes, and with prediabetes and newly diagnosed T2DM as secondary outcomes.

2. Methods

2.1. The Maastricht Study Design and Population

This study used data from the Maastricht Study, an observational prospective population-based cohort study. Its rationale and methodology have been described previously [19]. In brief, the study focuses on the etiology, pathophysiology, complications, and comorbidities of T2DM and is characterized by an extensive phenotyping approach. All individuals aged between 40 and 75 years and living in the southern part of The Netherlands were eligible for participation. Participants were recruited through mass media campaigns and from the municipal registries and the regional Diabetes Patient Registry via mailings. The representation with the source population in the study region was monitored continuously and was aligned with postal codes [19]. Recruitment was stratified according to known T2DM status, with an oversampling of individuals with T2DM, for reasons of efficiency. The study was approved by the institutional medical ethical committee (NL31329.068.10) and the Minister of Health, Welfare, and Sports of The Netherlands (Permit 131088-105234-PG). All participants gave written informed consent.

The present report includes cross-sectional data from the first 3451 participants who completed the baseline survey between November 2010 and September 2013. The examinations of each participant were performed within a time window of three months.

For the present analyses, individuals who had another type diabetes than type 2 (n = 41), suffered from cancer (n = 122), had not filled out a food frequency questionnaire (FFQ) (n = 154), or had reported implausible total energy intake (<800 or >4200 kcal/day for men and <500 or >3500 kcal/day for women, n = 63) were excluded [20]. Individuals with either a missing fasting or 120 min post glucose

load blood sample, or with overall less than five OGTT blood sampling points (n = 464) were excluded from the main analyses (primary outcomes: BCF and insulin sensitivity). This resulted in a study population of 2607 individuals for the primary outcome measures BCF and insulin sensitivity. For the analyses with newly diagnosed T2DM as an outcome, this study additionally excluded individuals with previously diagnosed T2DM (n = 738). For the analyses with prediabetes as an outcome, individuals with T2DM were excluded (n = 858). This resulted in a study population of 2213 and 2333 individuals for the secondary outcome measures prediabetes and newly diagnosed T2DM, respectively.

2.2. Glucose Metabolism

To determine glucose metabolism status, all participants—except those who used insulin—underwent a standardized 2 h 75 g oral glucose tolerance test (OGTT) after an overnight fast. Venous blood samples were collected before and 15, 30, 45, 60, 90, and 120 min after oral glucose load.

Plasma for the assessment of insulin and C-peptide levels was collected in ethylenediaminetetraacetic acid (EDTA) tubes, stored on ice, separated after centrifugation (3000× g for 15 min at 4 °C), and stored at −80 °C until the assays were performed. The time between collection and storage was <2 h. Insulin and C-peptide were measured in never-thawed plasma by use of a custom duplex array of MesoScale Discovery (MesoScale Discovery, Gaithersburg, MD, USA). In short, 96 well-plates, with capture antibodies against insulin and C-peptide patterned on distinct spots in the same well, were supplied by the manufacturer. Samples (10 μL/well), detection antibodies and read buffer for electrochemiluminescence were applied according to manufacturer's instruction and plates were read using a SECTOR® 2400 Imager. Detection ranges of the assay were 35–25,000 pg/mL for insulin and 70–50,000 pg/mL for C-peptide. Interassay coefficients of variation for insulin and C-peptide were 10.1% and 8.2%, respectively. Insulin and C-peptide values were converted from pg/mL to pmol/L using a molar mass of 5808 g for insulin and 3010 g for C-peptide.

Plasma for the assessment of glucose was collected in sodium fluoride/potassium oxalate (NaF/KOx) tubes on ice. Fasting and 120-min-postload plasma glucose were measured in fresh samples with the enzymatic hexokinase method by use of two automatic analysers (i.e., the Beckman Synchron LX20 (Beckman Coulter Inc., Brea, CA, USA) for samples obtained between November 2010 and April 2012, and the Roche Cobas 8000 (Roche Diagnostics, Mannheim, Germany) for samples obtained thereafter). Plasma for the assessment of glucose at other time points during the OGTT was separated after centrifugation (3000× g for 15 min at 4 °C) and stored within 2 h at −80 °C until the assays were performed. Glucose was measured in these never-thawed samples with the enzymatic hexokinase method by use of the Roche Cobas 6000 (Roche Diagnostics, Mannheim, Germany). The Pearson correlation coefficient between fresh and frozen samples were 0.96 and 0.99, respectively, for fasting and 120-min-postload plasma glucose samples (n = 486 samples) in a quality control.

Glucose metabolism status was defined according to the WHO 2006 criteria into normal glucose metabolism (NGM), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and T2DM [21]. Individuals without type 1 diabetes mellitus on diabetes medication were classified as having T2DM [19].

2.2.1. β-cell Function and Insulin Sensitivity

As BCF consists of multiple components, it cannot be described by a single measure. Therefore, we used three mathematical model-based parameters (β-cell glucose sensitivity, the potentiation factor ratio, and β-cell rate sensitivity) according to a previously described model [22], and two classic, relatively simple BCF-indices (C-peptidogenic index and the ratio of the C-peptide to glucose area under the curve) [22–25].

The mathematical model parameter "β-cell glucose sensitivity" is the slope of the glucose-insulin secretion dose-response function [22], and represents the dependence of insulin secretion on absolute glucose concentration at any time point during the OGTT. β-cell glucose sensitivity is a sensitive index to quantify β-cell dysfunction [13,26,27]. The dose-response relationship is modulated by

β-cell potentiation, which accounts for higher insulin secretion during the descending phase of hyperglycaemia than during the ascending phase of an OGTT for the same glucose concentration. β-cell potentiation is set as a positive function of time and averages 1 during the OGTT. Therefore, it represents the relative potentiation of the insulin secretion response to glucose. The β-cell potentiation parameter used in the present analysis represents the ratio of the β-cell potentiation factor at the end of the 2-h OGTT relative to the β-cell potentiation factor at the start. "β-cell rate sensitivity" is a marker of early phase insulin release, and represents the dynamic dependence of insulin secretion on the rate of change in glucose concentration [22].

The simple BCF-indices C-peptidogenic index ($\Delta CP_{30}/\Delta G_{30}$) and the ratio of the C-peptide to glucose area under the curve (CP_{AUC}/G_{AUC}) were also calculated. The C-peptidogenic index (the equivalent of the insulinogenic index) reflects early phase insulin secretion and has a good ability to discriminate between NGM and (pre)T2DM (ROC AUCs $\geq$ 78%) [23]. The CP_{AUC}/G_{AUC} is a measure of overall insulin secretion.

The Matsuda index ($10{,}000/\sqrt{G_0 \times I_0 \times \text{mean G} \times \text{mean I}}$) was used as a measure of insulin sensitivity [28].

2.2.2. Dietary Intake

All participants completed a food frequency questionnaire (FFQ) prior to being informed about their glucose metabolism status (e.g., NGM, prediabetes, or T2DM) [20].

Habitual dietary intake over the past 12 months was estimated by a tailor-made FFQ developed using the Dutch national FFQ tool [29]. The FFQ consisted of twenty-three product groups capturing 253 food items. Briefly, for each food item, frequency of consumption (ranging from "never or less than once a month" to "every day") and consumed amount were asked. Based on the FFQ data, daily intakes of the 23 main food product groups and nutrients were calculated (g/day), and subsequently daily nutrient intakes. The main food product groups were: 1. Bread (10 food items), 2. Savoury bread spreads (2 food items), 3. Cheese (9 food items), 4. Milk and milk products (45 food items), 5. Eggs (1 food item), 6. Cereals and cereal products (9 food items), 7. Soups (2 food items), 8, Potatoes (4 food items), 9. Vegetables (28 food items), 10. Legumes, (1 food item), 11. Meat, meat products and poultry (16 food items), 12. Fish (8 food items), 13. Soy and vegetarian products (7 food items), 14. Herbs and spices (3 food items), 15. Mixed dishes (9 food items), 16. Fats, oils, and savoury sauces (26 food items), 17. Fruits (20 food items), 18. Sugar, sweets, and sweet sauces (11 food items), 19. Nuts, seeds, and snacks (7 food items), 20. Pastry and biscuits (13 food items), 21. Alcoholic and non-alcoholic beverages (26 food items), 22. Clinical formulas (1 food item), and 23. Miscellaneous foods (1 food item).

Intakes of total energy and individual mono- and disaccharides (glucose, fructose, and sucrose) were calculated by using the Dutch Food Composition Database (NEVO) of 2011 [30]. Missing values for mono- and disaccharide contents of specific products in the NEVO database were substituted with values obtained from other relevant food composition tables [31]: first, McCance and Widdowson's "The Composition of Foods" for the UK [32], and second, if missing in this table, Fineli, the Finnish Food composition table [33] or the Danish Food Composition Table [34]. Individual mono- and disaccharides contents of food items not present in these international tables were imputed using equivalent food items or calculated using recipes included in the NEVO database or manufacturer's ingredient declarations.

2.2.3. Anthropometric and Other Measurements

Body height (cm) and weight (kg) were measured to the nearest 1 cm and 0.5 kg with the participants wearing light clothing and no shoes (Seca, Hamburg, Germany). Body mass index (BMI) was calculated as kilogram per meter squared (kg/m^2). Waist and hip circumference, blood pressure, and blood lipid profiles, including triglycerides, total cholesterol, and HDL and LDL cholesterol, were determined as described previously [19]. Finally, smoking status (never, former, or current smoker),

total and moderate-to-vigorous physical activity levels (hours/week), medication use, and history of cardiovascular disease (CVD) and cancer (yes/no) were self-reported. Detailed information concerning these measurements can be found in the study protocol of the Maastricht Study [19].

2.2.4. Statistical Methods

All analyses were performed using the software package SPSS Statistics version 23.0 for Windows (SPSS, IBM Corp., Armonk, NY, USA).

Characteristics of the study population were described as means and standard deviations (SD) for continuous variables or as number and proportions of participants per category for categorical variables (% of study population). The Matsuda index was log-transformed to obtain normally distributed data.

Linear regression analyses were performed to assess associations of glucose, fructose and sucrose intake with BCF indices (glucose sensitivity, potentiation factor, C-peptidogenic index, and overall insulin secretion) and the Matsuda index as continuous dependent variables. Logistic regression analyses were performed to assess the associations of mono- and disaccharide intakes with β-cell rate sensitivity, prediabetes, and T2DM. β-cell rate sensitivity was analyzed in tertiles (of which the highest tertile was considered the reference category) as the distribution of β-cell rate sensitivity was positively skewed and could not be normalized by transformations. Associations were presented as standardized regression coefficients (standardized betas (95% CI)) for continuous outcome measures and presented as odds ratios (OR (95% CI)) for categorical outcome measures.

The independent variables glucose, fructose, and sucrose were analyzed both categorically (comparing tertiles or quintiles) and continuously (per gram increment of glucose, fructose or sucrose intake). To test for linear trends across categories, an ordinal variable with the median value of individual mono- and disaccharide intake for each tertile or quintile was entered in the regression models.

To adjust for confounding, covariates were included in the regression models if their introduction in the model changed the regression coefficient of mono- and disaccharide intake by >10%. Regardless, model 1 was adjusted for sex and age. Model 2 was additionally adjusted for waist-to-hip-ratio (WHR), education level (low, middle, high), mean arterial blood pressure, CVD, anti-hypertensive medication, lipid-modifying medication, family history of T2DM, moderate-to-vigorous physical activity, and intake of total energy, dietary fibre, and alcohol. In our dataset, smoking status, total serum cholesterol, and the intake of saturated fat and *trans* fat did not confound the associations of the individual mono- and disaccharides intake with BCF, insulin sensitivity, prediabetes, and T2DM, and were therefore not included as covariates.

To assess BCF relative to the prevailing level of insulin resistance, the Matsuda index was included as a covariate in all regression models with a BCF index as the dependent variable.

Additional analyses were performed to evaluate the prediabetes subgroups IFG and IGT separately. Furthermore, to evaluate whether the associations of individual mono- and disaccharide intake with glucose metabolism were confounded by total energy intake and WHR, we additionally performed analyses without adjustment for total energy intake and WHR. Finally, analyses with BCF or insulin sensitivity as outcome measure were repeated after exclusion of individuals with previously diagnosed T2DM, as these persons might have adapted their dietary behavior.

3. Results

3.1. Population Characteristics

Of the 2818 participants included in this study, 1,757 individuals had NGM, 456 had prediabetes, 120 were newly diagnosed with T2DM, and 485 were previously diagnosed with T2DM. The mean (SD) age was 59.7 (8.16) years and 49.5% were male. The prevalence of overweight, obesity, or a high WHR (≥0.80 for women, ≥0.95 for men), hypertension, and history of CVD were lowest among

NGM individuals and highest among T2DM individuals. Previously diagnosed T2DM persons had the highest high-density lipoprotein:low-density lipoprotein (HDL:LDL) ratio, most likely due to lipid-modifying medication. BCF and insulin sensitivity were highest among NGM individuals and lowest among T2DM individuals. Moreover, self-reported glucose, fructose, and sucrose intakes were highest in the NGM group. No significant differences in total energy intake were observed between the groups (Table 1).

Table 1. Characteristics of study population.

	Total Population	NGM	Prediabetes	ND T2DM	PD T2DM	*p*-Value
	n = 2818	*n* = 1757	*n* = 456	*n* = 120	*n* = 485	
Sex (%male)	1395 (49.5)	743 (42.3)	243 (53.3)	75 (62.5)	334 (68.9)	<0.01
Age	59.7 ± 8.16	58.0 ± 8.08	61.7 ± 7.48	63.3 ± 7.49	62.9 ± 7.56	<0.01
BMI category (kg/m^2)	-	-	-	-	-	<0.01
<25	1048 (37.2)	844 (48.1)	113 (24.8)	20 (16.7)	71 (14.6)	-
25–30	1225 (43.5)	719 (40.9)	224 (49.1)	60 (50.0)	222 (45.8)	-
≥30	544 (19.3)	193 (11.0)	119 (26.1)	40 (33.3)	192 (39.6)	-
WHR cat (%normal)	576 (20.4)	466 (26.5)	68 (14.9)	14 (16.7)	28 (5.8)	<0.01
Systolic blood pressure (mmHg)	134 ± 17.8	131 ± 17.3	137 ± 16.7	143 ± 18.8	141 ± 16.8	<0.01
Diastolic blood pressure (mmHg)	76.3 ± 9.88	75.3 ± 9.94	77.9 ± 9.63	79.2 ± 10.2	77.8 ± 9.31	<0.01
CVD (%yes)	414 (15.1)	209 (12.2)	62 (13.9)	28 (23.5)	115 (24.9)	<0.01
Total PA (hours/week)	14.3 ± 8.07	14.9 ± 8.14	14.2 ± 7.78	13.2 ± 7.49	12.3 ± 7.92	<0.01
MVPA (hours/week)	5.64 ± 4.32	6.13 ± 4.40	5.17 ± 4.06	5.04 ± 4.08	4.33 ± 3.98	<0.01
Total energy intake (kcal)	2192 ± 607	2198 ± 608	2216 ± 586	2129 ± 599	2161 ± 627	0.33
Glucose (g/day)	17.3 ± 9.15	17.9 ± 9.54	16.4 ± 8.03	16.0 ± 7.49	16.2 ± 8.84	<0.01
Fructose (g/day)	19.0 ± 10.1	19.6 ± 10.4	18.2 ± 9.18	17.6 ± 8.35	18.1 ± 10.0	<0.01
Sucrose (g/day)	43.1 ± 24.5	44.3 ± 25.0	42.9 ± 23.6	42.5 ± 26.8	39.3 ± 22.4	<0.01
HbA1c (%)	5.73 ± 0.63	5.45 ± 0.34	5.71 ± 0.40	6.15 ± 0.62	6.68 ± 0.61	<0.01
HDL:LDL ratio	0.54 ± 0.26	0.54 ± 0.27	0.51 ± 0.23	0.48 ± 0.20	0.60 ± 0.29	<0.01
Triglycerides (mmol/L)	1.39 ± 0.82	1.22 ± 0.66	1.61 ± 1.06	1.89 ± 1.07	1.68 ± 0.85	<0.01
Glucose sensitivity (pmol/min/m^2/mM)	27.6 ± 18.5	33.3 ± 19.3	26.1 ± 12.3	17.8 ± 10.9	11.5 ± 7.97	<0.01
Rate sensitivity (pmol/m^2/mM)	250 ± 328	307 ± 376	237 ± 223	180 ± 239	81.1 ± 107	<0.01
Potentiation factor	1.63 ± 0.68	1.80 ± 0.73	1.55 ± 0.55	1.38 ± 0.43	1.16 ± 0.34	<0.01
CP_{AUC}:G_{AUC} ratio	214 ± 87.8	238 ± 81.8	215 ± 81.0	181 ± 81.5	136 ± 63.0	<0.01
ΔCP_{30}:G_{30} ratio	469 ± 1141	615 ± 1385	322 ± 683	303 ± 422	142 ± 113	<0.01
Matsuda index	4.04 ± 2.61	4.93 ± 2.65	2.92 ± 1.87	2.25 ± 1.35	2.52 ± 1.82	<0.01

n, number; BMI, body mass index; NGM, normal glucose metabolism; ND T2DM, newly diagnosed type 2 diabetes mellitus; PD T2DM, previously diagnosed type 2 diabetes mellitus; CVD, cardiovascular disease; PA, physical activity, MVPA, moderate-to-vigorous physical activity; HbA1C, glycated haemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Individuals with lowest glucose (tertile 1: <12.9 g) and fructose intakes (tertile 1: <14.1 g) were younger, were more often males, included more current smokers, had higher prevalences of overweight/obesity and prediabetes/T2DM, were less physically active, and consumed less fruits and vegetables compared to those individuals with highest intakes of glucose (tertile 3: ≥19.0 g) and fructose (tertile 3: ≥21.2 g). Individuals with the lowest intake of sucrose (tertile 3: <30.0 g) were older, had more often normal weight, were more often males and current smokers, had a higher prevalence of T2DM, and consumed less fruits and vegetables compared to participants that consumed the highest amounts of sucrose (tertile 3: ≥47.9 g).

3.2. Associations of Glucose, Fructose, and Sucrose Intake with BCF and Insulin Sensitivity

Glucose intake in the 3rd, 4th, and 5th quintiles were associated with higher β-cell glucose sensitivity, total insulin secretion, and insulin sensitivity, but not with β-cell potentiation factor, β-cell rate sensitivity, or C-peptidogenic index in age- and sex-adjusted models (Tables 2 and 3). The association between glucose intake and insulin sensitivity remained statistically significant in the fully adjusted model, with a standardized β (95% CI) of 0.07 (0.00, 0.14) for the 5th vs. the 1st quintile (Table 2). Glucose intake was not associated with BCF.

Table 2. Associations of mono- and disaccharide intake with continuous insulin sensitivity and β-cell function (BCF) measures (standardized betas and 95% confidence intervals).

			Continuous	Quintiles Mono- and Disaccharides					P_{trend}
				1 (ref)	2	3	4	5	
				β	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	
B-cell glucose sensitivity	Glucose			<10.19 g	10.19–13.91 g	13.91–17.58 g	17.58–22.79 g	≥22.79 g	-
		1	0.02 (−0.02, 0.05)	0	0.03 (−0.01, 0.08)	0.05 (0, 0.09)	0.05 (0, 0.10)	0.04 (−0.01, 0.09)	0.1
		2	0.01 (−0.05, 0.07)	0	0.03 (−0.03, 0.09)	0.02 (−0.05, 0.08)	0.02 (−0.05, 0.09)	0.06 (−0.02, 0.13)	0.26
	Fructose			<10.86 g	10.86–15.27 g	15.27–19.67 g	19.67–25.51 g	≥25.51 g	-
		1	0 (−0.04, 0.04)	0	0.03 (−0.02, 0.07)	0.04 (−0.01, 0.07)	0.03 (−0.02, 0.08)	0.02 (−0.03, 0.07)	0.44
		2	−0.02 (−0.08, 0.05)	0	−0.01 (−0.08, 0.05)	0.01 (−0.06, 0.07)	−0.02 (−0.09, 0.05)	0.01 (−0.07, 0.09)	1
	Sucrose			<23.36 g	23.36–33.25 g	33.25–43.61 g	43.61–58.74 g	≥58.74 g	-
		1	0.04 (0.01, 0.08)	0	0.06 (0.01, 0.10)	0.06 (0.01, 0.10)	0.07 (0.02, 0.12)	0.06 (0.01, 0.11)	0.05
		2	0.05 (−0.02, 0.12)	0	0.03 (−0.04, 0.09)	0.04 (−0.03, 0.10)	0.09 (0.02, 0.16)	0.05 (−0.03, 0.13)	0.12
B-cell potentiation factor	Glucose			<10.19 g	10.19–13.91 g	13.91–17.58 g	17.58–22.79 g	≥22.79 g	-
		1	0 (−0.04, 0.04)	0	−0.02 (−0.07, 0.03)	−0.02 (−0.07, 0.03)	−0.03 (−0.07, 0.02)	−0.01 (−0.06, 0.04)	0.76
		2	0.04 (−0.02, 0.09)	0	0.01 (−0.06, 0.07)	−0.02 (−0.09, 0.05)	0 (−0.07, 0.08)	0.03 (−0.05, 0.11)	0.64
	Fructose			<10.86 g	10.86–15.27 g	15.27–19.67 g	19.67–25.51 g	≥25.51 g	-
		1	0 (−0.04, 0.04)	0	−0.02 (−0.06, 0.03)	−0.02 (−0.07, 0.03)	−0.01 (−0.06, 0.04)	−0.02 (−0.06, 0.03)	0.62
		2	0.05 (−0.02, 0.12)	0	0.01 (−0.06, 0.08)	0 (−0.07, 0.07)	0.02 (−0.05, 0.09)	0.03 (−0.05, 0.11)	0.7
	Sucrose			<23.36 g	23.36–33.25 g	33.25–43.61 g	43.61–58.74 g	≥58.74 g	-
		1	−0.03 (−0.06, 0.01)	0	0.02 (−0.03, 0.07)	0.01 (−0.04, 0.06)	0.03 (−0.02, 0.08)	−0.02 (−0.07, 0.03)	0.36
		2	−0.06 (−0.13, 0.01)	0	0 (−0.07, 0.07)	0.02 (−0.05, 0.09)	0.06 (−0.01, 0.13)	−0.02 (−0.11, 0.06)	1
C-peptidogenic index	Glucose			<10.19 g	10.19–13.91 g	13.91–17.58 g	17.58–22.79 g	≥22.79 g	-
		1	0 (−0.04, 0.04)	0	0.03 (−0.02, 0.08)	0.04 (−0.01, 0.09)	−0.01 (−0.06, 0.04)	0.03 (−0.02, 0.08)	0.63
		2	−0.03 (−0.10, 0.05)	0	0.03 (−0.04, 0.10)	0.05 (−0.03, 0.12)	−0.04 (−0.12, 0.04)	0.02 (−0.07, 0.11)	0.8
	Fructose			<10.86 g	10.86–15.27 g	15.27–19.67 g	19.67–25.51 g	≥25.51 g	-
		1	0 (−0.04, 0.04)	0	0.01 (−0.04, 0.06)	0 (−0.05, 0.05)	−0.02 (−0.06, 0.04)	0.02 (−0.03, 0.07)	0.9
		2	−0.02 (−0.09, 0.05)		−0.01 (−0.08, 0.07)	0.01 (−0.07, 0.08)	−0.04 (−0.12, 0.04)	0.01 (−0.08, 0.10)	0.89
	Sucrose			<23.36 g	23.36–33.25 g	33.25–43.61 g	43.61–58.74 g	≥58.74 g	-
		1	0.02 (−0.02, 0.06)	0	0.02 (−0.03, 0.07)	−0.01 (−0.06, 0.04)	0.02 (−0.03, 0.07)	0.02 (−0.03, 0.07)	0.38
		2	−0.01 (−0.08, 0.07)	0	0 (−0.07, 0.08)	−0.05 (−0.12, 0.03)	−0.01 (−0.09, 0.07)	−0.01 (−0.10, 0.09)	0.93

Table 2. *Cont.*

			Continuous	Quintiles Mono- and Disaccharides					
				1 (ref)	2	3	4	5	P_{trend}
				β	β (95% CI)	β (95% CI)	β (95% CI)	β (95% CI)	
Overall insulin secretion	Glucose			<10.19 g	10.19–13.91 g	13.91–17.58 g	17.58–22.79 g	≥22.79 g	-
		1	0.01 (−0.02, 0.05)	0	0.06 (0.02, 0.11)	0.04 (−0.01, 0.09)	0.09 (0.04, 0.13)	0.04 (−0.01, 0.08)	0.19
		2	−0.03 (−0.09, 0.03)	0	0.03 (−0.03, 0.09)	−0.02 (−0.08, 0.05)	0.03 (−0.03, 0.10)	0.01 (−0.07, 0.08)	0.97
	Fructose			<10.86 g	10.86–15.27 g	15.27–19.67 g	19.67–25.51 g	≥25.51 g	-
		1	0 (−0.04, 0.04)	0	0.01 (−0.04, 0.06)	0.02 (−0.03, 0.07)	0.02 (−0.03, 0.07)	0.01 (−0.04, 0.05)	0.78
		2	−0.05 (−0.11, 0.01)	0	−0.03 (−0.09, 0.03)	−0.03 (−0.09, 0.03)	−0.03 (−0.10, 0.03)	−0.03 (−0.10, 0.05)	0.47
	Sucrose			<23.36 g	23.36–33.25 g	33.25–43.61 g	43.61–58.74 g	≥58.74 g	-
		1	0.05 (0.01, 0.09)	0	0.05 (0.01, 0.10)	0.06 (0.01, 0.11)	0.08 (0.03, 0.12)	0.05 (0.01, 0.10)	0.06
		2	−0.01 (−0.07, 0.06)	0	0.01 (−0.06, 0.07)	0.01 (−0.05, 0.07)	0.05 (−0.02, 0.11)	−0.02 (−0.09, 0.06)	0.83
Insulin sensitivity	Glucose			<10.19 g	10.19–13.91 g	13.91–17.58 g	17.58–22.79 g	≥22.79 g	-
		1	0.10 (0.07, 0.14)	0	0 (−0.04, 0.05)	0.06 (0.02, 0.11)	0.05 (0, 0.10)	0.10 (0.05, 0.14)	<0.01
		2	0.10 (0.05, 0.16)	0	0.01 (−0.04, 0.07)	0.01 (−0.05, 0.07)	0.04 (−0.02, 0.10)	0.07 (0, 0.14)	0.03
	Fructose			<10.86 g	10.86–15.27 g	15.27–19.67 g	19.67–25.51 g	≥25.51 g	-
		1	0.09 (0.06, 0.13)	0	0.01 (−0.04, 0.06)	0.03 (−0.02, 0.08)	0.04 (−0.01, 0.09)	0.08 (0.04, 0.13)	<0.01
		2	0.08 (0.03, 0.14)	0	0.02 (−0.03, 0.08)	−0.01 (−0.07, 0.05)	0.01 (−0.05, 0.07)	0.06 (−0.01, 0.13)	0.12
	Sucrose			<23.36 g	23.36–33.25 g	33.25–43.61 g	43.61–58.74 g	≥58.74 g	-
		1	0.01 (−0.03, 0.04)	0	0.01 (−0.04, 0.06)	0.04 (−0.01, 0.09)	0.02 (−0.03, 0.07)	0 (−0.05, 0.05)	0.97
		2	0.01 (−0.05, 0.07)	0	0 (−0.05, 0.06)	0.03 (−0.03, 0.09)	0.01 (−0.06, 0.07)	0.03 (−0.04, 0.10)	0.32

CI, confidence interval. Positive values indicate a better β-cell function (BCF) or insulin sensitivity, negative values indicate a lower BCF or insulin sensitivity. M1: sex, age, insulin sensitivity. M2: M1 + waist-to-hip ratio, cardiovascular diseases, blood pressure expressed in mean arterial pressure, lipid-modifying medication, antihypertensive medication, family history of type 2 diabetes mellitus (T2DM), moderate-to-vigorous physical activity, total intake of energy, and dietary fibre and alcohol intake.

Table 3. Associations of mono- and disaccharide intake with β-cell rate sensitivity tertiles (odds ratios and 95% confidence intervals).

		Tertile 1 vs. 3 of β-Cell Rate Sensitivity					Tertile 2 vs. 3 of β-Cell Rate Sensitivity				
		Continuous	Tertiles Mono- and Disaccharides				Continuous	Tertiles Mono- and Disaccharides			
			1 (ref)	2	3	P_{trend}		1 (ref)	2	3	P_{trend}
			OR	OR (95% CI)	OR (95% CI)			OR	OR (95% CI)	OR (95% CI)	
			<12.76	12.76–18.99 g	≥18.99 g	-		<12.76	12.76–18.99 g	≥18.99 g	-
Glucose	1	1.00 (0.99, 1.01)	1	0.92 (0.73, 1.16)	0.82 (0.65, 1.04)	0.06	1.00 (0.99, 1.01)	1	1.02 (0.81, 1.29)	0.91 (0.72, 1.15)	0.37
	2	1.00 (0.98,1.02)	1	1.03 (0.74, 1.43)	0.87 (0.59, 1.29)	0.47	1.00 (0.98, 1.02)	1	1.09 (0.79, 1.50)	0.80 (0.55, 1.18)	0.24
			<13.94 g	13.94–21.17 g	≥21.17 g	-		<13.94 g	13.94–21.17 g	≥21.17 g	-
Fructose	1	1.00 (0.99, 1.01)	1	0.86 (0.68, 1.08)	0.86 (0.68, 1.09)	0.18	1.00 (0.99, 1.01)	1	1.03 (0.82, 1.30)	0.90 (0.71, 1.14)	0.34
	2	1.00 (0.98, 1.02)	1	0.92 (0.66, 1.29)	0.86 (0.58, 1.27)	0.42	1.00 (0.99, 1.02)	1	1.08 (0.78, 1.48)	0.83 (0.57, 1.21)	0.24
			<29.98	29.98–47.64 g	≥47.64 g	-		<29.98	29.98–47.64 g	≥47.64 g	-
Sucrose	1	1.00 (1.00, 1.00)	1	0.79 (0.63, 1.00)	0.90 (0.71, 1.15)	0.38	1.00 (1.00, 1.00)	1	0.87 (0.70, 1.10)	0.88 (0.69, 1.12)	0.30
	2	1.00 (0.99, 1.01)	1	0.97 (0.69, 1.35)	1.00 (0.66, 1.50)	0.45	1.00 (1.00, 1.01)	1	0.97 (0.70, 1.34)	1.07 (0.72, 1.59)	0.86

CI, confidence interval. Third tertile of β-cell rate sensitivity is the reference group (best rate sensitivity). Values <1.00 indicate a better β-cell rate sensitivity, values >1.00 indicate a lower β-cell rate sensitivity. M1: sex, age, insulin sensitivity. M2: M1 + waist-to-hip-ratio, cardiovascular diseases, blood pressure expressed in mean arterial pressure, lipid-modifying medication, antihypertensive medication, family history of T2DM, moderate-to-vigorous physical activity, total intake of energy, and dietary fibre and alcohol intake.

Fructose intake was associated with higher insulin sensitivity for the 5th vs. the 1st quintile in the age- and sex-adjusted model. This association was attenuated in the fully adjusted model with a β of 0.06 (−0.01, 0.13). No associations of fructose intake with BCF were found (Tables 2 and 3).

Sucrose intake was associated with higher β-cell glucose sensitivity and total insulin secretion in the age- and sex-adjusted models for the 2nd, 3rd, 4th, and 5th quintiles vs. the 1st quintiles. In the fully adjusted model, the association for the 4th vs. the 1st quintile of sucrose intake with β-cell glucose sensitivity remained significant with a β of 0.09 (0.02, 0.16) (Table 2). Results revealed no significant associations of sucrose intake with other BCF indices and insulin sensitivity.

3.3. Associations of Glucose, Fructose, and Sucrose Intake with Prediabetes and T2DM

Glucose intake in the 4th and 5th quintiles were associated with a lower odds of prediabetes in the fully adjusted model with ORs (95% CI) of 0.48 (0.28, 0.81) for quintile 4 vs. quintile 1, and of 0.50 (0.28, 0.90) for quintile 5 vs. quintile 1 (Table 4).

Table 4. Associations of mono- and disaccharide intake with prediabetes and T2DM (odds ratio and 95% confidence intervals).

		Continuous	**Quintiles Mono- and Disaccharides**					P_{trend}
			1 (ref)	**2**	**3**	**4**	**5**	
			OR	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	**OR (95% CI)**	
					Prediabetes			
			<10.61 g	10.61–14.43 g	14.43–18.16 g	18.16–23.66 g	≥23.66 g	-
Glucose	1	0.98 (0.96, 0.99)	1	0.95 (0.69, 1.30)	0.78 (0.56, 1.08)	0.58 (0.41, 0.81)	0.61 (0.44, 0.85)	<0.01
	2	0.97 (0.94, 0.99)	1	0.78 (0.50, 1.22)	0.85 (0.53, 1.37)	0.48 (0.28, 0.81)	0.50 (0.28, 0.90)	<0.01
			<11.14 g	11.14–15.72 g	15.72–20.11 g	20.11–26.43 g	≥26.43 g	-
Fructose	1	0.98 (0.97, 0.99)	1	1.02 (0.74, 1.41)	0.97 (0.70, 1.33)	0.63 (0.45, 0.90)	0.71 (0.51, 0.99)	<0.01
	2	0.98 (0.96, 1.00)	1	1.02 (0.64, 1.63)	1.10 (0.68, 1.77)	0.82 (0.48, 1.40)	0.83 (0.46, 1.48)	0.29
			<24.53 g	24.53–34.50 g	34.50–45.30 g	45.30–61.58 g	≥61.58 g	-
Sucrose	1	1.00 (0.99, 1.00)	1	0.87 (0.63, 1.21)	0.94 (0.68, 1.30)	0.78 (0.56, 1.10)	0.86 (0.62, 1.21)	0.33
	2	1.00 (0.99, 1.01)	1	1.00 (0.63, 1.59)	0.87 (0.54, 1.42)	0.68 (0.41, 1.15)	0.71 (0.40, 1.26)	0.13
		Continuous	**Tertiles mono- and disaccharides**					P_{trend}
			1 (ref)	**2**	**3**			
			OR	**OR (95% CI)**	**OR (95% CI)**			
					T2DM			
			<13.10 g	13.10–19.49 g	≥19.49 g			-
Glucose	1	0.98 (0.95, 1.00)	1	0.97 (0.62, 1.51)	0.75 (0.47, 1.19)			0.18
	2	0.98 (0.93, 1.02)	1	1.72 (0.87, 3.37)	1.05 (0.44, 2.52)			0.87
			<14.34 g	14.34–21.64 g	≥21.64 g			-
Fructose	1	0.98 (0.96, 1.00)	1	1.01 (0.65, 1.54)	0.61 (0.37, 0.99)			0.06
	2	0.97 (0.94, 1.01)	1	1.35 (0.70, 2.59)	0.54 (0.22 (2.86)			0.43
			<31.10 g	31.10–49.36 g	≥49.36 g			-
Sucrose	1	1.00 (0.99, 1.01)	1	0.84 (0.54, 1.32)	0.81 (0.51, 1.28)			0.37
	2	1.01 (0.99, 1.02)	1	1.26 (0.64, 2.48)	1.00 (0.43, 2.31)			0.98

CI, confidence interval. Values <1.00 indicate a lower odds of prediabetes or T2DM, values >1.00 indicate a higher odds of prediabetes or T2DM. M1: sex, age. M2: M1 + waist-to-hip ratio, cardiovascular diseases, blood pressure expressed in mean arterial pressure, lipid-modifying medication, antihypertensive medication, family history of T2DM, moderate-to-vigorous physical activity, total intake of energy, dietary fibre and alcohol intake.

High fructose intake was associated with lower odds of prediabetes and T2DM as compared to low fructose intake in the age- and sex-adjusted models. These associations did not remain statistically significant in fully adjusted models (Table 4).

Sucrose intake was not significantly associated with prediabetes or T2DM in any of the models (Table 4).

3.4. Additional Analyses

Analysis of associations for subcategories of prediabetes, e.g., IFG (n = 123) and IGT (n = 333), revealed that higher intake of glucose was associated with a lower odds of IGT, with ORs of 0.51 (0.28, 0.92) for quintile 4 vs. quintile 1, and 0.51 (0.26, 0.99) for quintile 5 vs. quintile 1 in the fully

adjusted model (Table S1). Furthermore, when glucose intake was included as a continuous variable, a gram increment of glucose intake was associated with lower odds of both IFG (ORs: 0.94 (0.90, 0.99)) and IGT (OR: 0.97 (0.95, 1.00)) in the fully adjusted models. No significant associations were found for fructose and sucrose intakes with IFG and IGT.

Analyses with the exclusion of all confounders except total energy intake and WHR yielded essentially the same results (Tables S2–S4), except for the association of fructose and insulin sensitivity, which was attenuated after adjustment for WHR (β 0.06 (−0.01, 0.13)).

Exclusion of individuals with previously diagnosed T2DM did not materially affect the results for the primary outcomes BCF and insulin sensitivity (Tables S5 and S6).

4. Discussion

This study's novelty is the evaluation of the associations of glucose, fructose, and sucrose intake with multiple indices of pancreatic BCF and insulin sensitivity. In the fully adjusted models, a positive association of glucose, but not of fructose and sucrose intake, was found with insulin sensitivity. No clear associations were observed for glucose, fructose, and sucrose intake with BCF.

The positive association of glucose intake with insulin sensitivity has been reported previously [16]. In contrast with the absence of an association between fructose intake and insulin sensitivity in this study, one study [16] showed a positive association, and another study [15] showed an inverse association of fructose intake with insulin sensitivity. Lau et al. [16] did not take dietary fibre intake into account, whereas in our data, adjustment for dietary fibre intake diminished the associations of glucose and fructose intake with insulin sensitivity. A possible explanation for the difference in association between this study and the study of Coello et al. [15] might be the high average fructose intake in their population (mean ± SD: 48.6 ± 31.0 g/day), which might have undermined the health benefits of fruit fibre. Higher intake of sucrose was associated with favourable β-cell glucose sensitivity, but not with insulin sensitivity. The latter finding is in agreement with findings of others [16].

The majority of existing studies focus on the associations of glucose, fructose, and sucrose intake with prediabetes and T2DM. The results of these previous studies are mixed. In the present study, glucose intake was inversely associated with prediabetes. An inverse association of fructose with prediabetes prior to adjustments (Table 3, age- and sex-adjusted model) was observed which proved to be confounded mainly by fibre intake. The absence of an association between fructose and prediabetes in this study contrasts with the positive association observed in another study [4]. However, the positive association observed in that previous study only appeared significant after additional adjustment for dietary fibre [4]. Therefore, it is suggested that dietary fibre may partly account for the association of fructose, derived from fruit and vegetable sources, and glucose metabolism [6,16]. An inverse association of sucrose intake with prediabetes has been reported before [8] but was absent in this study. The absence of an association of glucose, fructose, and sucrose with T2DM found in this study is in agreement with some previous findings [5,7,9,10] but not with all previous studies [2,5,6].

Some previous studies suggest that the beneficial associations of glucose and fructose intake with insulin sensitivity might be confounded by a healthy dietary behaviour, including a high consumption of fibre, fruits, and vegetables [6,35]. Indeed, fruit and vegetables were main sources of glucose and fructose intake in our study. The dietary fibre, vitamins, and antioxidants in fruit and vegetables might improve glucose metabolism [6,36–38]. As mentioned before, in this study, the association of glucose intake with insulin sensitivity was attenuated, and the association of fructose intake with insulin sensitivity became nonsignificant after adjusting for dietary fibre. A beneficial role of fructose may also be explained by the relatively low glycaemic response, partly caused by its low glycaemic index, which may result in a lower burden on the β-cells, lower insulin resistance, and a decreased risk of T2DM [39,40]. Another potential explanation for a beneficial association between mono- and disaccharide intake and glucose metabolism is the tendency of obese persons to underreport their mono- and disaccharides consumption [41]. However, previous studies [6,8] and stratified analyses

in the current study indicate that the beneficial associations of mono- and disaccharide intake with glucose metabolism were only present in non-obese individuals (BMI < 30 kg/m^2).

Other studies suggest that higher intake of glucose, fructose, and sucrose unfavourably affect glucose metabolism through increased energy intake and a subsequent higher WHR [42]. However, as in the case of a previous study [7], this study's results did not confirm this; the associations between glucose, fructose, and sucrose with BCF, insulin sensitivity, prediabetes, and T2DM were independent of total energy intake and WHR. Regarding glucose intake, the relatively high glycemic index may result in a higher burden on the β-cells, higher insulin resistance and an increased risk of T2DM [5,40]. Finally, concerning fructose intake, it is suggested that the increased fatty acid level causes insulin resistance [42], which results in a higher burden on β-cells [42].

Inconsistent findings across studies might be explained by the difference in main sources of mono- and disaccharide intake, as the effects of added vs. naturally occurring sugars might differ [7]. The consumption of mono- and disaccharides through fruit and vegetable intake might be beneficially associated with glucose metabolism, whereas added mono- and disaccharides consumption might be associated with worse glucose metabolism [35]. Furthermore, the number of food items within each product group differs in the FFQs used in individual studies. Hence, the degree by which sources of mono- and disaccharide are measured by FFQs might differ. Regarding the FFQ used in the current study, covered nutrient intake of mono- and disaccharide intake was 98% (Eussen et al. submitted), which reflects an almost complete assessment of the relevant food items.

One of the strengths of this study is that β-cell dysfunction and insulin resistance provide physiological information on early abnormalities in glucose metabolism that eventually result in prediabetes and T2DM. Furthermore, the continuous outcome measures of BCF and insulin sensitivity prevent individuals to be misclassified as normal or abnormal glucose metabolism. Another strength is the use of multiple indices to assess various aspects of BCF, and the use of non-fasting BCF and insulin sensitivity indices which better reflect metabolic conditions. In addition, the large sample size and the inclusion of participants covering the total glucose metabolism spectrum from NGM to T2DM, and the extensive data collection on demographics and lifestyle factors, allowed a thorough adjustment for confounders. However, a major limitation is the cross-sectional design of our study, which formally rules out causal inference. Nevertheless, all participants completed the FFQ before being informed about their glucose metabolism status, which minimized the risk of reporting bias. Furthermore, previously diagnosed T2DM were excluded in analyses with T2DM as outcome measure, and exclusion of previously diagnosed T2DM in analyses with BCF or insulin sensitivity as outcome measures did not materially affect the results (see Tables S5 and S6). This makes it unlikely that dietary adaptations caused by glucose metabolism status influenced the results.

Prospective studies will be needed to corroborate these findings. Furthermore, as this is the first study focused on the association of glucose, fructose, and sucrose with non-fasting insulin sensitivity and multiple BCF aspects, more studies are needed to evaluate these potential associations and to investigate the specific mechanisms involved. Finally, because there might be differences in effects of mono- and disaccharide sources on BCF, insulin sensitivity, and glucose metabolism status, future studies should evaluate the associations of added versus natural sources of glucose and fructose intake with BCF, insulin sensitivity, and glucose metabolism status.

5. Conclusions

In the current cross-sectional population-based cohort study, high glucose intake was associated with higher insulin sensitivity and a decreased odds of prediabetes, independent of dietary fibre. There is no convincing evidence for associations of glucose, fructose, and sucrose intake with BCF. The predictive effects of mono-and disaccharides on BCF, insulin sensitivity, and T2DM risk still need to be confirmed in future prospective cohort studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/4/380/s1, Table S1: Associations of mono- and disaccharides with IFG and IGT, Table S2: Associations of mono- and

disaccharides with BCF and insulin sensitivity, with and without adjustment for total EI and WHR, Table S3: Associations of mono- and disaccharides with β-cell rate sensitivity, with and without adjustment for total EI and WHR, Table S4: Associations of mono- and disaccharides with prediabetes and T2DM, with and without adjustment for total EI and WHR, Table S5: Associations of mono- and disaccharides with BCF and insulin sensitivity, with exclusion of persons with PD T2DM, Table S6: Associations of mono- and disaccharides with β-cell rate sensitivity, with exclusion of persons with PD T2DM.

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nutrients

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Review

Dietary Sugars and Endogenous Formation of Advanced Glycation Endproducts: Emerging Mechanisms of Disease

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Abstract: The rapid increase in metabolic diseases, which occurred in the last three decades in both industrialized and developing countries, has been related to the rise in sugar-added foods and sweetened beverages consumption. An emerging topic in the pathogenesis of metabolic diseases related to modern nutrition is the role of Advanced Glycation Endproducts (AGEs). AGEs can be ingested with high temperature processed foods, but also endogenously formed as a consequence of a high dietary sugar intake. Animal models of high sugar consumption, in particular fructose, have reported AGE accumulation in different tissues in association with peripheral insulin resistance and lipid metabolism alterations. The in vitro observation that fructose is one of the most rapid and effective glycating agents when compared to other sugars has prompted the investigation of the in vivo fructose-induced glycation. In particular, the widespread employment of fructose as sweetener has been ascribed by many experimental and observational studies for the enhancement of lipogenesis and intracellular lipid deposition. Indeed, diet-derived AGEs have been demonstrated to interfere with many cell functions such as lipid synthesis, inflammation, antioxidant defences, and mitochondrial metabolism. Moreover, emerging evidence also in humans suggest that this impact of dietary AGEs on different signalling pathways can contribute to the onset of organ damage in liver, skeletal and cardiac muscle, and the brain, affecting not only metabolic control, but global health. Indeed, the most recent reports on the effects of high sugar consumption and diet-derived AGEs on human health reviewed here suggest the need to limit the dietary sources of AGEs, including added sugars, to prevent the development of metabolic diseases and related comorbidities.

Keywords: advanced glycation end products; fructose; glucose; lipogenesis; sphingolipids; NLRP3; Nrf2; mitochondrial dysfunction; oxidative stress

1. Dietary Sugars as a Risk for Health

1.1. Sugar Consumption in Modern Society

Significant modifications of human diet composition, as well as of frequency and timing of energy and nutrients intake, have been observed in the last forty years, representing potential risk factors for the development of metabolic diseases. An increase of the daily energy intake of 505 kcal, corresponding to 25%, from 1970 to 2010 [1] has been described, and a rise in *per capita* food consumption from 5 kg to 70 kg per year from 1800 to 2006 has been estimated [2]. Based on these observations, the current Guidelines of Nutrition and Health Recommendations suggest that a healthy diet must provide no more than 5% of total energy intake as simple sugars. In contrast, currently, 13% of the American population consumes over 25% of their daily energy intake as sugar [3].

In addition, clinical evidence suggests that sugar-sweetened foods create psychological dependence [4]. Indeed, clinical observations report that removing sugar from the diet causes effects like hyperactivity, conduct problems, and mental disturbances [5,6].

On the other hand, results from experimental models confirm that the consumption of sugar-added foods is associated with increased risk for obesity [7], as well as cardiovascular diseases [8,9], metabolic disorders [10], non-alcoholic fatty liver disease (NAFLD) [11,12], and cognitive decline [13]. Actually, some controversial conflicts over the role of an excessive intake of sweetened foods and beverages on public health and the interest of food and beverage industry have been debated [14]. Recent reviews report several critical issues on the criteria and low quality evidence used for recommendations and guidelines [15–17].

However, sugar added to foods and drinks adds considerable calories without any benefits and may take the place of other nutrient-dense foods in the diet. Thus, many of the clinical and epidemiological observations indicating that excess glucose and fructose intake exacerbates metabolic complications in different tissues are possibly due to the increased calories intake. At present, there seems to be reliable evidence of results obtained from experimental models about the negative effects of high dietary sugar intake, but no clear reliable evidence indicating daily caloric thresholds for sugar intake to exert negative health effects in human.

1.2. Fructose Consumption and Prevalence of Metabolic Diseases

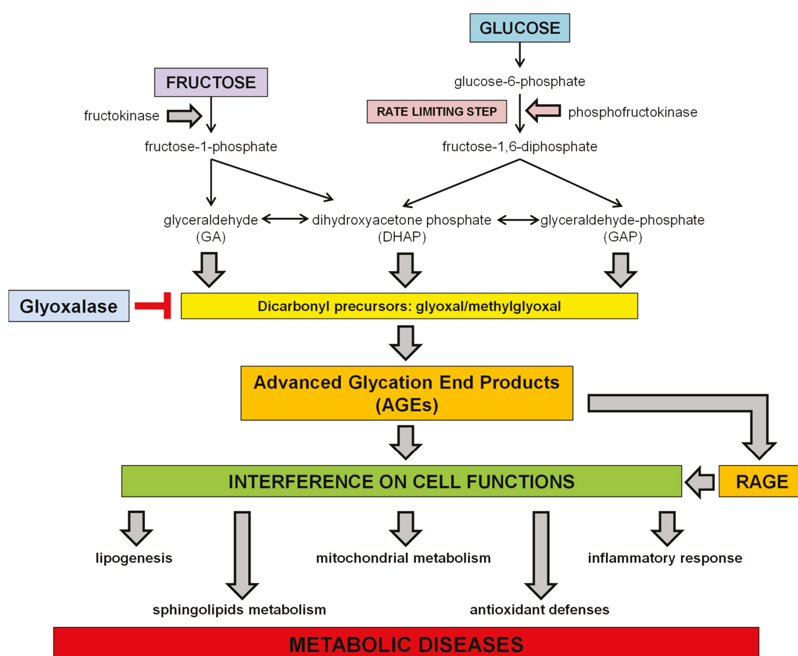
Sucrose, formed by 50% fructose and 50% glucose joint by a glycosidic bond, has been the most easily consumed sweetener in the last decade. The introduction of corn-derived sweeteners, in particular of high-fructose corn syrup (HFCS), which is provided with high sweetening power, organoleptic properties, the ability to confer a long shelf life and to maintain a long-lasting hydration in industrial bakeries, together with its low cost, has rapidly reduced the use of sucrose in many industrial preparations [2]. The fructose content in HFCS is in a range between 42% and 55% of total sugar, and both fructose and glucose are in their pure form, without glycosidic bond. The commercial use of HFCS as a common sweetener has strongly raised the content of fructose in the human diet through consumption of sweetened beverages, tea, coffee, sodas, snacks, and bakeries.

Some epidemiological studies show an association between fructose-containing sweeteners intake and body weight gain [8,18]. Moreover, clinical evidence indicates that a high-fructose diet is associated with the onset of dyslipidemia, insulin resistance, and related metabolic diseases [19,20]. These observations in humans have been confirmed and further extended by animal studies indicating that fructose added to the diet contributes to the development of obesity, inflammation, and decrease of the activity of the mitochondrial metabolism regulator peroxisome proliferator-activated receptor alpha (PGC1-alpha) [10,21]. High-sugar fed animals are commonly used as suitable experimental models to highlight pathogenic mechanisms related to metabolic diseases onset following imbalanced high-calories diets [22–24]. However, although the negative effects of fructose have been observed and described in these models, the mechanisms proposed are not yet exhaustive to define whether dietary fructose, when consumed in moderate amounts, is actually deleterious for human health.

1.3. Dietary Fructose and Glucose Metabolism

Dietary sugars, including glucose and fructose, are absorbed in the small intestine, but the absorptive capacity for fructose is lower than for glucose or sucrose. However, the addition of glucose, as in case of HFCS-added foods, facilitates the absorption of fructose [25]. After absorption, nevertheless, the metabolism of the two monosaccharides follows different pathways, since glucose can be used directly by the cells to produce energy in a variety of organs, while fructose is primarily metabolized in the liver, which takes up at least 50% of the initial fructose flux [26]. In the cells, glucose is phosphorylated by hexokinase to glucose-6-phosphate, which is then converted to fructose-1-6-diphosphate by the phosphofructokinase, the rate limiting enzyme of glycolysis. The cleavage of fructose-1,6-diphosphate by fructose diphosphate aldolase produces the triose

phosphate intermediates dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). Conversely, the fructose metabolism bypasses the need of insulin and the phosphofructokinase regulation step, and enters glycolysis directly at the step of triose phosphate intermediates generation. Indeed, fructose is phosphorylated by ATP to fructose-1-phosphate, catalyzed by fructokinase. Fructose-1-phosphate is then split by hepatic aldolase B into glyceraldehyde (GA) and DHAP, which can be both converted to GAP. These metabolites are at the centre of metabolic crossroads that lead to glycolysis, gluconeogenesis, glycogenesis and lipogenesis [27] (see Scheme 1).



Scheme 1. Fructose and glucose metabolism and proposed effects for dietary sugar-induced glycation. Fructose bypasses the rate limiting step of glycolysis leading to a faster generation of dicarbonyl precursors than glucose. AGE accumulation in tissues can interfere with many protein functions contributing to the onset of metabolic diseases and related comorbidities.

2. Dietary Sugars and Glycation

2.1. Advanced Glycation Endproducts (AGEs)

AGEs are toxic compounds deriving from non-enzymatic glycoxidation reactions of reducing sugars with proteins, which then result as being structurally and functionally compromised [28]. Protein glycation is initiated by a nucleophilic addition reaction between the free amino group from a protein, lipid or nucleic acid and the carbonyl group of monosaccharides. This reaction forms a reversible Schiff base, which rearranges over a period of days to produce ketoamine or Amadori products. The Amadori products undergo dehydration and rearrangements followed by other reactions involving dicarbonyl compounds, such as cyclization, oxidation and dehydration, to form irreversible AGEs [29]. Proteins glycation occurs in vivo in physiological conditions and the Maillard reaction represents a type of post-translational modification of molecules that takes place slowly and continuously throughout the life span, driving AGE accumulation in tissues during

ageing [20]. For this reason, AGEs have been involved in the pathogenesis of age-related diseases, such as neurodegenerative diseases, atherosclerosis, and chronic inflammatory diseases [30], but in particular conditions, such as diabetes and insulin resistance, the accumulation of AGEs is accelerated, leading to early developing of comorbidities [31]. Indeed, hyperglycemia is known to induce high rates of protein glycation, which is responsible for the development of long-term complications [30].

2.2. In Vitro Protein Glycation of Different Sugars

It has been a long time since studies started to investigate the glycative potential of different monosaccharides by in vitro incubations with physiologically relevant proteins such as haemoglobin and serum albumin [32,33]. The first comparison among sugars for their non-enzymatic reactivity with haemoglobin has been published by Bunn and Higgins in 1981, showing that fructose has a reaction rate 7.5-fold higher than glucose [32]. A few years later, Suarez et al. found that the rate of glycoxidation of bovine serum albumin by fructose was about 10 times higher than that by glucose [34]. Since then, other studies have confirmed the sequence ribose > fructose > glucose for the rate of glycoxidation [35,36], even if some of them failed to detect relevant differences on the glycation potential of fructose compared to glucose in terms of time and intensity of browning during incubation with amino acids [37,38].

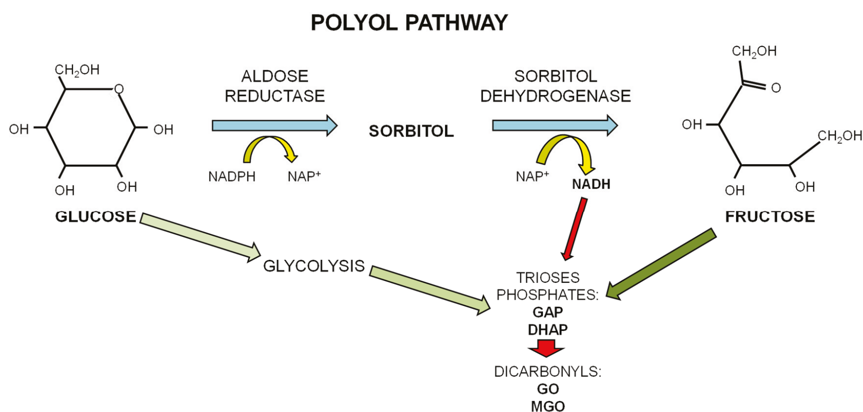
The papers reporting a different kinetic in AGE formation from glucose and fructose have attributed it to the different forms in which the two sugars exist in physiological conditions. Glucose, a D-aldohexose, exists in solution as a stable ring structure. Since only the open chain form of sugar can react with an amino group in protein to form a Schiff base, the high reactivity of fructose may reflect its higher quote of existing as an open chain in solution than glucose [32,33]. The main glycating sugar present in the body at the highest concentration is glucose. However, fructose can be produced in conditions of hyperglycemia by the polyol pathway where glucose is converted to fructose through the consecutive action of aldose reductase and sorbitol dehydrogenase. Oxidation of sorbitol by sorbitol dehydrogenase (SDH) yields NADH and causes an increase in the ratio NADH/NAD+ that may contrast GAP-dehydrogenase activity, thus leading to accumulation of the triose phosphates (see Scheme 2). This conversion of excessive glucose to fructose leads to an increase in fructose levels in tissues of diabetic patients [39]. A second aspect linking high fructose levels to AGE production is related to the peculiar fructose metabolism that evokes rapid generation and accumulation of GAP and DHAP, both effective glycating agents and precursors of the dicarbonyls compounds glyoxal and methylglyoxal, which, in turn, are precursors of more stable AGEs [40].

There are differences between glucose and fructose also concerning the glycating process, since the rearrangement of the Schiff base derived from fructose generates Heyns products that are quite different from the Amadori products formed by glucose, for they undergo a more rapid conversion to irreversible AGEs [34]. However, in addition, the opposite observation of a slower rate of Maillard fluorescence generation for the Haynes products derived from fructose compared to Amadori products derived from glucose has been reported [41].

2.3. Dietary Intake of Exogenous AGEs and of Simple Sugars as Potential Sources for Endogenous AGEs

In recent years, several studies have highlighted some dietary aspects that can influence extra- and intra-cellular accumulation of AGEs.

First, the high-temperature and long-time cooking of foods can generate AGEs that are exogenously introduced with the diet [42]. A relevant number of intervention studies, reviewed by Kellow and Savige, have investigated the effects of an AGE-restricted diet on inflammatory markers and insulin sensitivity. Their meta-analysis of the literature provided evidence that, although the reduction of AGEs introduced with foods is associated to reduced CML plasma levels, a direct relationship with improved insulin sensitivity and inflammatory profile is still not clearly demonstrated by high quality clinical trials [43].



Scheme 2. The polyol pathway. In conditions of excess of glucose, as occurring in diabetes, glucose undertakes the polyol pathway to be converted to fructose through the consecutive action of aldose reductase and sorbitol dehydrogenase. This alternative pathway for the metabolism of glucose leads to the increase in fructose levels in tissues of diabetic patients and of the NADH/NAD+ ratio that contrasts GAP-dehydrogenase activity leading to accumulation of the triose phosphates GAP and DHAP, precursors of the dicarbonyls compounds glyoxal (GO) and methylglyoxal (MGO).

Second, emerging evidence indicates that high dietary simple sugars consumption can represent a substantial source of endogenous AGEs [44]. In light of the in vitro observation that fructose is much more reactive than glucose in generating glycation precursors, the modern nutrition implicating a much higher fructose intake than decades ago is an important factor contributing to the increase in plasma fructose concentration in healthy subjects [45], possibly contributing to AGE formation. Thus, among the sugars mostly used for sweetening of foods and drinks, the fructose might represent the most hazardous for AGE accumulation. Population studies have evidenced that, in individuals with NAFLD, the concomitant presence of metabolic syndrome is related to the consumption of sugar-sweetened beverages [46,47] and, in particular, a high intake of fructose-containing drinks and foods in the general population is associated with induction of lipogenesis with hypertriglyceridemia, and insulin resistance, paralleled by oxidative stress, which is a relevant factor contributing to the glycation process [48–50]. Indeed, plasma and tissue AGE accumulation has been reported in animal models of high fructose consumption [12,51], while experimental data on in vivo fructose-derived glycation consequences are still limited.

3. Dietary Sugar-Induced Glycation: Interference on Cell Functions

In addition to the effects attributed to the excess of calorie intake induced by high sugars consumption on metabolism, the sugar-derived AGEs have been shown in animal models to contribute to the development of pathological metabolic conditions through the interference with several protein functions and the activation of pro-inflammatory signals [52,53], as illustrated above in Scheme 1.

3.1. Interference with the SCAP/SREBP Lipogenic Pathway

Several studies have demonstrated that excessive fructose consumption is associated with increased ectopic lipid deposition in liver and skeletal muscle both in experimental models and in humans [48,49,54,55], and that fructose is able to promote hepatic lipogenesis [56,57] by inducing the expression and activation of transcription factors including sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) [52,58,59].

A positive correlation between lipogenesis and plasma triglyceride has been described in patients with isocaloric feeding added with high fructose level [60].

Besides the alterations of plasma lipid profile, fructose is suggested to be an important risk factor for the development of NAFLD [11]. The pathogenic mechanisms related to intrahepatic fat content induced by fructose consumption are related to an imbalance among fatty acid synthesis, beta-oxidation and triglyceride outflow from the liver [24]. Indeed, the direct comparison between saturated fats- and fructose-rich diets revealed that these two dietary components differently affect liver lipid metabolism, with fructose enhancing both beta-oxidation and fatty acids export, counteracted by a strong activation of lipogenesis and palmitate production [53].

The fructose-induced ectopic lipid deposition has been attributed to the activation of the transcription factor SREBP1c, which regulates the expression of several enzymes responsible for fatty acids de novo synthesis [61]. In studies directly comparing the effect of pure fructose and glucose in lipid metabolism a greater impact of fructose on lipogenesis activation has been demonstrated [48]. In particular, the chronic consumption of fructose- and glucose-sweetened beverages in mice revealed activation of the SCAP/SREBP pathway was, to a greater extent, in fructose-drinking mice [12]. The lipogenesis activation was associated with a different pattern of AGEs in the plasma and liver of sugar-drinking mice, with a higher amount of glyoxal derived AGEs, such as glyoxal-lysine dimer (GOLD) and carboxymethyl lysine (CML), which are more resistant to AGE-degrading enzymes, in the livers of the fructose group. The hypothesis that excessive intake of dietary sugars might interfere with lipid metabolism through the action of AGEs has been confirmed by the observation that the SREBP-activating protein SCAP is highly glycated by CML, a modification evoking prolonged activation of SCAP by inhibiting its degradation [12,52,62].

The same mechanism has been proposed to enhance intramyocellular lipid deposition in the skeletal muscle of high-fructose consuming mice [52]. Interestingly, in skeletal muscle of mice, the overactivation of lipogenesis induced by high fructose intake was accompanied by modifications of muscle metabolic reprogramming and mitochondrial functions that were effectively reverted by the administration of an anti-glycative agent, demonstrating the relevant impact of fructose-derived AGEs on tissue specific signaling pathways.

3.2. Interference on Sphingolipids Metabolism

The deregulated enhancement of the de novo lipid synthesis can have relevant repercussions on the overall lipids metabolism. Recently, alterations of the sphingolipid metabolism have been evidenced in obese and diabetic patients, with increased plasma levels of ceramide and sphingosine-1-phosphate, which are hypothesized to elicit an inflammatory condition [63,64]. In animal models of the western diet, high in fats and fructose, increased ceramide de novo synthesis has been reported, in relation with reduced insulin sensitivity [65,66]. In parallel, two very recent studies highlighted a possible role for diet-derived AGEs on this unbalance among sphingolipid intermediates through the action of the AGE-receptor RAGE [67,68]. In particular, according to the work from Geoffrey and colleagues, the dose- and time-dependent effect of exposition to AGEs on mesangial cells proliferation was mediated by the modulation of the sphingolipids intermediates ceramide/sphingosine/sphingosine-1-phospahate and activities of related enzymes [67]. Similarly, the findings from Chen et al. demonstrated that polydatin, through its anti-glicative effect, evokes the reduction of sphingosine kinase activity and its byproduct sphingosine-1-phosphate, and this may be the underlying mechanism for the prevention of diabetic nephropathy and glomerular mesangial cells' pro-fibrogenic signalling [68]. The pro-inflammatory profile induced by high levels of ceramide and sphingosine-1-phosphate has been demonstrated to contribute to cardiac impairment and peripheral insulin resistance, and the modulation of enzymes involved in their accumulation has been shown to ameliorate metabolic derangements [69,70]. However, the beneficial effects of prevention of AGE accumulation induced by high fructose intake on sphingolipids metabolism remains to be explored.

3.3. Interference on Inflammatory Response

Since the last few years, there has been strong evidence for a causative association between central (visceral) obesity and the development of type 2 diabetes and cardiovascular complications, though the mechanisms are not fully understood. However, in this context, a low-grade, chronic inflammation orchestrated by metabolic cells in response to excess nutrients and energy in metabolic tissues including adipose, liver, muscle, pancreas, and brain, has been defined as a causative factor underlying metabolic dysfunctions development and related comorbidities [71].

Besides indications that a high fructose intake is also associated with the onset of a generic inflammatory response in several tissues, recent studies have addressed the specific activation of the NLRP3 inflammasome complex in models of a high-fructose diet [25,53,72]. In a comparative study performed on mice fed a normal or a western-style diet, associated or not to fructose-sweetened beverages, the peculiar effect of fructose drinking was the renal activation of NLRP3 inflammasome [73]. Indeed, the targeting of inflammasome activation by antioxidant and antidiabetic compounds or selective NLRP3 inhibitors, has been demonstrated to be effective in reducing the inflammatory response in kidneys, hearts, livers, skeletal muscles, and brains of mice fed a fructose-containing diet [74–78]. The inflammasomes are multiporotein platforms activated by interaction of a variety of danger signals with membrane and cytoplasmic receptors. Among these receptors, a role for two AGE receptors, namely RAGE and Galectin-3, in inflammasome activation has been proposed [79–82]. However, there is still contrasting data about the exact mechanisms by which both Galectin-3 and RAGE act on inflammasome assembling. If the pro-inflammatory effect of RAGE signalling through activation of the NFkB pathway is already well known [83], whether Galectin-3 exerts a positive or negative stimulus for NLRP3 activation is still under debate [84,85]. In this perspective, further investigations are needed to definitely clarify whether the fructose-induced inflammasome activation is mediated by AGE accumulation and to understand the involved mechanisms.

3.4. Interference with Mitochondrial Function and Oxidative Stress

Mitochondrial dysfunction and oxidative stress are strictly interconnected events representing the common features of metabolic disorders and chronic inflammatory diseases. It has been reported that a high intake of dietary sugars can evoke a mitochondrial overload in tissues with a high rate of energy metabolism, such as liver and cardiac and skeletal muscle, leading to enhanced mitochondrial respiratory chain activity and oxidative stress [65,86,87]. In particular, complexes I and III being the key-point for reactive oxygen species production, the enhancement of their activity due to increased energy influx results in oxidative stress, which, in turn, can compromise the activity of the iron-sulfur center enzymes, such as the complex I and II, and the complex III itself [88]. It is well known that oxidative species favour the glycoxidation reaction of proteins in the presence of reducing sugars, thus resulting in AGE accumulation. In turn, AGEs exert a pro-oxidant effect compromising antioxidant enzymes activity and mitochondrial functions, thus creating a vicious cycle [89]. In this regard, a very recent study evidenced that fructose feeding activates in rats the so-called AROS axis, featured by the consecutive enhancement in plasma AGEs level—tissue RAGE activation—mitochondrial ROS production, with subsequent intracellular AGE formation [44]. In particular, recent research has provided evidence that overconsumption of carbohydrates in the diet, especially sugars, may represent a risk factor for neurodegenerative diseases through the development of mitochondrial dysfunction, oxidative stress, and inflammatory reaction, and the cerebral accumulation of AGEs is considered a key mediator [90–92].

In addition, further recent findings highlighted in models of high-fructose intake the impairment of the transcriptional activity of the nuclear factor erythroid 2-related factor 2 (Nrf2), a central player in the regulation of many antioxidant enzymes, including glyoxalase-1, the main enzyme responsible for AGE detoxification [53,92,93], suggesting a twofold contribution of fructose on glycation through both enhanced production and reduced detoxification of AGEs.

4. Dietary Sugar-Induced Glycation and Pathogenic Role in Diseases

4.1. Data from Animal Studies

As mentioned above, the glycation induced by dietary sugars, through interference with many cell functions and signalling pathways, may contribute to the development of tissue damage and organ disease. However, most research studies on the pathogenic role of endogenous sugar-derived glycation have been performed in animal models, often through the employment of very high doses of sugars, particularly of fructose, not comparable to the general human nutrition.

Notably, in animals, the metabolic outcomes of a caloric restriction were attenuated by the diet enrichment with methyl glyoxal, demonstrating that dietary AGEs can induce oxidative stress, insulin resistance, and profibrotic conditions independently from total calorie amount [94]. In addition, in animal models, an involvement of AGEs specifically derived from dietary sugars in metabolic disturbances or in organ dysfunction has been demonstrated by the use of anti-glycative agents. Betanin, an antioxidant compound also provided with anti-glycative properties in vitro, prevented in rats' hearts the collagen cross-link and expression of markers of fibrosis that were increased after 60 days of drinking 30% fructose solution [95]. In a very recent work, the administration to rats of the PPARγ agonist Rosiglitazone was able to improve most of the signs of metabolic syndrome induced by a 60% fructose drinking for 21 days, through the reduction of urine and plasma AGE levels and of kidney and liver RAGE expression [44]. Our research group has previously reported the beneficial effects of a specific anti-glycative compound, pyridoxamine, in a mouse model of metabolic syndrome induced by a 12-week 60% fructose diet, where the prevention of AGE accumulation in plasma, skeletal muscle, and brain was paralleled by an improvement of systemic glucose and lipid metabolism and reduction of inflammatory and oxidative stress markers and restoration of mitochondrial function in skeletal muscle and brain [52,92]. Pyridoxamine administration has also been found to be effective in the amelioration of glucose homeostasis and insulin sensitivity, and in the prevention of visceral adipose tissue expansion and local expression of inflammatory markers in a mouse model of high-fat diet, suggesting an involvement of diet-induced endogenous formation of AGEs in the pathogenesis of obesity [96]. In this regard, in mice with genetically-induced deletion of leptin receptors $Db^{-/-}$), which are prone to consume excessive calories and develop obesity and insulin resistance, high levels of CML were trapped by the adipose tissue, while the deletion of RAGE reverted CML accumulation in adipose tissue, increasing its plasma levels, indicating a RAGE-dependent mechanism underlying endogenous AGE-induced obesity [97].

4.2. Data from Human Studies

On the other hand, epidemiological and observational studies in humans have not univocally demonstrated a relation between high sugar intake and organ diseases, such as liver steatosis, cardiac impairment, or neurodegeneration, so far. Excessive soft drink consumption has only been associated with increased risk for metabolic disorders such as obesity and insulin resistance [98,99], but their effects are often attributed to an unspecific calorie excess [100,101]. In this regard, two recent studies revealed that an isocaloric fructose restriction, where the calories of fructose were substituted by starch, was sufficient to improve metabolic parameters and lipoprotein markers of CVD risk, in particular reducing apoC-III, which has been associated with atherogenic hypertriglyceridemia, in children with obesity and metabolic syndrome, irrespective of weight change, indicating that the detrimental effects of sugar, specifically fructose, are independent of its caloric value or effects on adiposity [102,103]. Moreover, a very recent double-blind randomized crossover trial analysed acute metabolic and endocrine responses induced by fructose and glucose load in healthy young subjects and showed that fructose leads to unfavorable modifications of some metabolic parameters, including increased serum concentrations and 3 h-AUC of uric acid, aldose reductase, and lactic dehydrogenase, increased systolic blood pressure, and decreased endothelial nitric oxide production in comparison with the same amount of glucose [104].

Nevertheless, the involvement of endogenous glycation in dietary sugar-induced dysmetabolism is far from being demonstrated in humans. Though, the reduction of dietary (exogenous) AGEs in type 2 diabetes or obese patients, without modification of the total calories intake, has also been demonstrated to be effective in amelioration of insulin sensitivity, with reduction of inflammatory markers and restoration of AGEs detoxifying systems and mitochondrial metabolism regulators [42,105]. In this regard, to induce the expression and activity of the AGE-detoxyfying enzyme glyoxalase-1, through the synergic action of trans-resveratrol and hesperetin, is likely to be a promising strategy to prevent the onset of insulin resistance and vascular inflammation in overweight individuals [106].

Moreover, endogenous and exogenous AGEs have been related to cognitive decline and impaired memory in two different studies, indicating that serum levels of methylglyoxal in elderly individuals were directly correlated with dietary AGE intake and cognitive decline assessed by the Mini Mental State Examination [107,108].

Recent cross-sectional studies from DeChristopher and colleagues indicated that the consumption of HFCS or fructose-sweetened beverages is associated with asthma and bronchitis in adults and to asthma in children [109–111]. Authors suggest an interesting mechanistic hypothesis, according to the in vitro observations of Bains and Gugliucci [112], of an intestinal formation of AGEs from excess free fructose intake, which may be absorbed in the circulation and induce a systemic inflammatory condition through the binding to RAGE, thus contributing to lung diseases and impaired immune response. This fascinating theory, described in detail in the very recent review from Gugliucci [113], however, is still not supported by in vivo experimental data.

5. Conclusions

In the present review, we provide experimental data and epidemiological observations indicating the negative effects of excessive intake of sugar-added foods and beverages, particularly of fructose. The data here reported also suggest that the glycation process following high sugar intake may play a central role in the development of metabolic disturbances by interfering with many cell signaling pathways and influencing the pro-inflammatory and pro-oxidant status that contributes to tissue injury and organ dysfunction.

However, these mechanisms need to be deepened through animal models trying to mimic the real human sugar consumption and aimed to clarify the significance of dietary sugar-derived AGEs in metabolic diseases and increase the transferability to the human nutrition.

Nevertheless, it is difficult to definitely establish limits for sugar intake above which risks for human health are increased. The guidelines to limit sugar addition in foods are not always free of conflicts of interest between public health and the food industry. Thus, since to date it is not possible to discriminate in human nutrition the contribution of different monosaccharides and the general calorie excess to protein glycation, high quality clinical trials are needed to evaluate the sugar daily intake, particularly of fructose, that can represent a feasible risk for human health, through the design of appropriated dietary interventions.

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Differential Effect of Sucrose and Fructose in Combination with a High Fat Diet on Intestinal Microbiota and Kidney Oxidative Stress

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Abstract: There is controversial information about the adverse effect of sucrose (S) or fructose (F) in the development of obesity. Thus, the purpose of the study was to evaluate the effect of S or F in a high fat diet (HF) on gut microbiota and renal oxidative stress. Rats were fed for four months with either high-fat + sucrose (HFS) or high-fat + fructose (HFF) or a control diet (C). Half of the HFS or HFF groups were maintained with the same diet and the other half were switched to the consumption of C. HFS and HFF groups increased 51% and 19% body weight, respectively, compared with the C group. Body fat mass, metabolic inflexibility, glucose intolerance, lipopolysaccharide (LPS), insulin, renal reactive oxygen species (ROS), malondialdehyde (MDA), *Nadphox*, and *Srebp-1* were significantly higher and antioxidant enzymes and lean body mass were significantly lower in the HFS group with respect to the HF-F group. Change in the consumption of HFS or HFF to a C diet ameliorated the insulin and glucose intolerance. The type of carbohydrate differentially modified the microbiota composition, however, both groups significantly decreased *C. eutactus* with respect to the C group. Thus, metabolic alterations with the HFS diet had a more detrimental effect than HFF.

Keywords: microbiota; renal oxidative stress; sucrose; fructose; obesity; LPS; inflammation

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1. Introduction

Since 1980, overweight and obesity have more than doubled in the worldwide population [1]. In addition to the decrease of physical activity in a genetically-stable population, increased consumption of foods with a high content of energy, mainly attributed by sugar and fat, constitutes the main environmental factor, contributing to this dramatic increase in obesity that has reached epidemic levels [2]. Obesity is often accompanied by hyperinsulinemia and, consequently, dyslipidemia, which could impact the renal structures, like the glomerulus, contributing to vasodilation and hypertension, leading to kidney injury [3].

In recent years, the modification of gut microbiota and activation of inflammation pathways have been implicated in the development of insulin resistance and renal disease related to obesity [4]. Kidney disease is associated with low fiber consumption and increased intestinal permeability due to a modified intestinal microbiota [5]. During obesity there is an inflammatory state and imbalance in the gut microbiota composition (dysbiosis) involving the toll-like receptor (TLR) family. Obesity results in an increase of circulating lipopolysaccharide (LPS) because of the disruption of the enterocyte junctions resulting in increased gut permeability [4]. Another mechanism involved in the kidney

injury is the protein fermentation of certain bacteria that result in an increase in uremic toxins, such indoxyl sulfate and *p*-Cresyl sulfate [6]. TLR-4 is an important component of the innate immune system signaling through different ligands that recognize, among others, LPS, associated with kidney disease [7,8]. Saturated fatty acids [8], fructose [9,10], or sucrose can activate inflammatory pathways observed in obesity and kidney injury [7,8]. LPS, in turn, induces insulin resistance through the activation of the transcription factor NF-κB (nuclear factor kappa-beta) which promotes the expression of inflammatory cytokines [4], such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), which contribute to the impairment of insulin signaling, affecting kidney function [11]. The production of these cytokines leads to a redox imbalance and an increase in reactive oxygen species (ROS), establishing a continuous cycle of inflammation and oxidative stress [12–14] implicated in chronic kidney disease (CKD). Interestingly, a recent study suggests that obesity and kidney damage are associated with insulin-resistant obese individuals. During insulin resistance there is an increased production of radicals and a decrease of some antioxidant enzymes [12,15]. ROS impairs insulin signaling by inhibiting GLUT 4 translocation to the cell membrane [16,17]. The increase in ROS concentration induces lipid peroxidation that contributes to oxidative stress with malondialdehyde (MDA) and the accumulation of ROS in the blood as end products [13,14]. There is sufficient information about the effect of sucrose or fructose on obesity [18] and its consequences on inflammation and oxidative stress [19]. However, part of the increase in obesity, worldwide, is caused by the consumption of a high fat diet and sugars [2], yet there is less information available about the differential effects of sugars in combination with a high fat diet on gut microbiota and kidney damage [20].

It is still not understood whether the consumption of a high fat diet and different kinds of sugars can cause differential effects on diet-induced obesity. Thus, the purpose of the present study was to assess the long-term effects (four months) of a high fat diet in combination with ad libitum access to one of two sweetened solutions (5% sucrose or 5% fructose) on the onset of obesity and metabolic abnormalities that impact the intestinal microbiota and the kidney oxidative stress on Wistar rats.

2. Materials and Methods

2.1. Animals

Male Wistar rats aged 5–7 weeks were obtained from the National Institute of Medical Sciences and Nutrition. The animals were housed in individual cages and maintained at a controlled room temperature with 12-h light-dark cycles and free access to water and food. The study was developed in two stages. In the first stage rats were randomized into three groups; eight rats were fed a high-fat diet and 5% sucrose in drinking water (HFS), eight rats were fed a high-fat diet and 5% fructose in drinking water, and eight rats were fed a control diet (C) [21] for four months. In the second stage, four rats fed with HFS or four rats HFF were changed to the control diet, (HFS-C) and (HFF-C), respectively (n = 4 per group) for two months. The control group continued consuming the control diet (n = 8). Animal weight and food consumption were determined every other day during the protocol. At the end of the study urine and stool of each rat were collected and stored at −70 °C until analysis. At the end of the experiment, the rats were killed by decapitation after being anesthetized with CO_2. The kidney was rapidly removed and stored at −70 °C until analysis. Serum was obtained by centrifugation of blood at 1500× g for 10 min and stored at −70 °C until further analysis. The Animal Committee of the National Institute of Medical Sciences and Nutrition, Mexico City (CINVA1444) approved the procedure.

2.2. Gut Microbiota Profiling

Fresh feces samples were collected immediately, frozen, and stored at −70 °C until use. Bacterial DNA content was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Bacterial DNA was amplified by PCR with barcode universal bacterial primers targeting variable regions 3–4 of the 16S rRNA gene. We used the specific forward and reverse primers: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACGGGTATCTAATCC-3′. Samples were pooled and sequenced with the Illumina MiSeq platform (MiSeq Reagent Kit V.3, 600 cycles, San Diego, CA, USA) according to the protocol suggested by Illumina (16S metagenomic sequencing library preparation).

Sequence processing

For taxonomic composition analysis, custom C# and Python scripts, as well as Python scripts in the Quantitative Insights Into Microbial Ecology (QIIME, San Diego, CA, USA) software pipeline 1.9, were used to process the sequencing files. The sequence outputs were filtered for low-quality sequences (defined as any sequences that are <200 bps or >600 bps, sequences with any nucleotide mismatches to either the barcode or the primer, sequences with homopolymer runs >6, sequences with an average quality score of <25, and sequences with ambiguous bases >0) and were truncated at the reverse primer. Sequences were chimera checked with Chimera Slayer, and chimeric sequences were filtered out. Analysis started by clustering sequences within a percent sequence similarity into operational taxonomic units (OTUs) with a 97% similarity threshold. Thus, 99.68%, 98.1%, 98.08%, 82.74%, 56.43%, and 14.82% of the reads were assigned to the phylum, class, order, family, genus, and species levels, respectively. Species richness (Observed, Chao1) and alpha diversity measurements (Shannon) were calculated, and we estimated the within-sample diversity at a rarefaction depth of 5495 reads per sample. Weighted and unweighted UniFrac distances were used to perform the principal coordinate analysis (PCoA). Differences in the relative abundance at the phylum, family, genus, and species levels were compared using a Student's *t*-test for independent samples.

2.3. Biochemical Parameters

Serum biochemical parameters including glucose, triglycerides, total, and LDL cholesterol were analyzed with a COBAS C11 (Roche, Basel, Switzerland). Serum insulin (Alpco Diagnostics, Salem, NH, USA) and LPS (Cloud-Clone Corp, Houston, TX, USA) were determined using commercial ELISA kits.

2.4. Glucose Tolerance Test

The glucose tolerance test was determined as previously reported [22] by the administration of an intraperitoneal injection of a glucose load of 2 g per kg body weight in fasted rats. The blood samples were collected from the tail vein at 0, 15, 30, 45, 60, 90, and 120 min after administration of the glucose. Plasma glucose concentration was measured using a OneTouch Ultra Glucose Meter (LifeScan, Inc., Milpitas, CA, USA)

2.5. Energy Expenditure

Energy expenditure was determined by indirect calorimetry in an Oxymax Lab Animal Monitoring (CLAMS) System (Columbus Instruments, Columbus, OH, USA). The animals were individually housed in plexiglass cages with an open-flow system connected to the CLAMS. Throughout the test, O_2 consumption (VO_2 mL/kg/h) and CO_2 production (VCO_2, mL/kg/h) were measured sequentially for 90 s. The respiratory exchange ratio (RER) was calculated as the average ratio of VCO_2 produced to VO_2 inhaled (VCO_2/VO_2).

2.6. Western Blot Analysis

Total protein of pooled kidney samples (n = 4) was extracted and quantified by Bradford assay (Bio-Rad, Hercules, CA, USA) and stored at −70 °C. The protein detection was performed by

electrophoresis in SDS-PAGE and then transferred to polyvinylidene difluoride membranes. All blots were blocked with 5% nonfat dry milk (Bio-Rad, Hercules, CA, USA) for 60 min at room temperature and incubated overnight at 4 °C with the following antibodies: toll-like receptor 4 (TLR-4) (1:10,000), nuclear factor-kappa B (NF-κB) (1:3000), and tumor necrosis factor α/β (TNF-α) (1:2000). The blots were incubated with anti-rabbit, anti-goat, or anti-mouse secondary antibodies conjugated with horseradish peroxidase (1:3500). Actin (1:5000) was used to normalize the data. Images were analyzed with a ChemiDocTM XRS + System Image LabTM Software (Bio-Rad, Hercules, CA, USA). The assays were performed three times using independent blots.

2.7. Renal Gene Expression

The gene expression was determined by real-time PCR. First, total RNA was extracted using TRIzol, following the manufacturer's instructions. The mRNA abundance was measured by real-time quantitative PCR using Taqman or SYBR® Green assays (Applied Biosystems, Foster, CA, USA), using *HPRT* and *cyclophilin* as references for normalization (Table 1).

Table 1. Primers used in real-time PCR.

Gene	Primer (5′-3′)	Sequence
Srebp-1c	Forward Reverse	CGTTGTACTGCAGCCACACT AGTGGTACTGTGGCCAGGAT
Nadph oxidase	Forward Reverse	GTCCCTTTGGCACAGTCAGT AGGCACCCGTCTCTCTACAA
ucp-1	Forward Reverse	CCGAAACTGTACAGCGGTCT TGACCTTCACCACCTCTGTG
tlr-4	Forward Reverse	GTGCCCCGCTTTCAGCTTTG GTGCCTCCCCAGAGCATTGT
Il-1beta	Forward Reverse	CAGCAGCATCTCGACAAGAG CATCATCCCACGAGTCACAG
Il-6	Forward Reverse	ACCACCCACAACAGACCAGT CGGAACTCCAGAAGACCAGA
hprt	Forward Reverse	CTGGTGAAAAGGACCTCTCG GGCCACATCAACAGGACTCT
Cyclophilin	Forward Reverse	CGTGGGCTCCGTTGTCTT TGACTTTAGGTCCCTTCTTCTTATCG
Fluorogenic probes TaqMan		
Catalase (Cat)		Rn01423343_m1
Glutathione peroxidase (Gpx)		Rn00588153_m1
Glutathione reductase (Gr)		Rn99999088_g1
Superoxide dismutase 1 (Sod1)		Rn00560930-m1

2.8. Oxidative Markers

Reactive oxygen species (ROS) in kidney were measured by a fluorescence method. A 50 μL homogenate was incubated with 150 μL of fluorescent compound 5-(and-6) carboxy-2,7-dichlorofluorescein (carboxy-DCF) (5 μM) for 1 h at 37 °C. Fluorescence was measured in a Synergy HT multimode microplate reader (Biotek, Winooski, VA, USA). The data were expressed as fluorescence units/mg of protein [23]. MDA concentration was measured spectrophotometrically. A solution of 1-methyl-2-phenylindole was diluted in acetonitrile: methanol (3:1) was added to 300 μL of kidney homogenate, then 150 μL of HCl (37%) were added and incubated for 40 min at 45 °C and the optical density was measured at 586 nm. Data were expressed as nmol MDA/mg protein [24]. The hydrogen peroxide in urine was measured by using Amplex red. The assay was performed with 25 μL of urine and 50 μL of the reaction mixture (0.1 mM Amplex Red, HRP 0.2 U mL^{-1}) and incubated for 30 min in the dark at room temperature. Finally, the fluorescence intensity was measured in a Synergy HT multimode microplate reader (Biotek, Winooski, VA, USA). The results were expressed by μM/mL of urine.

2.9. Statistical Analysis

The results were expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-hoc test, using Prism 5.0 software (GraphPad, San Diego, CA, USA); $p < 0.05$ was considered significant.

3. Results

3.1. Body Composition and Energy Expenditure

After six months, the HFS and HFF groups showed 55% and 19% weight gain with respect to the C group (Figure 1A). When the HFS and HFF groups were switched for two months to the control diet, there was a significant decrease in body weight by 22% and 7%, respectively. Body weight gain was related with changes in the body composition. Remarkably, 51% of the body in the HFS group was fat mass and only 24% was lean mass, whereas the HFF group had 40% fat mass and 48% lean mass, indicating that the presence of sucrose in the diet produced a more deleterious effect than the fructose in the development of fat mass (Figure 1B,C). These adverse effects were, in part, reverted by the consumption of an adequate diet for rodents (Figure 1B,C). These alterations in body composition were accompanied by metabolic alterations. The HFS group showed marked metabolic inflexibility, i.e., the inability to switch substrates during the feeding period, with lipids being the main substrate, whereas the HFF group showed a less severe metabolic inflexibility compared with C group (Figure 1D, $p < 0.05$). These changes were associated with lower oxygen consumption (Figure 1E).

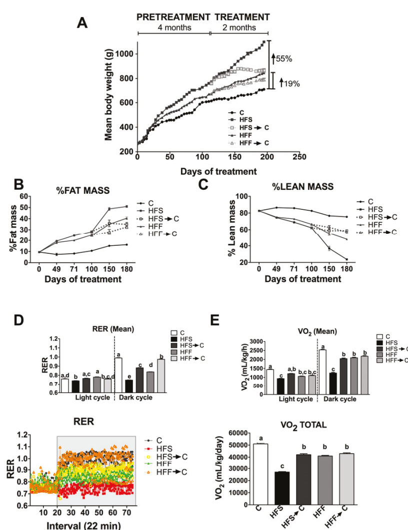

Figure 1. Effect of a high fat diet enriched with 5% of sucrose (HFS) or fructose (HFF) in water on body weight (**A**); fat mass (**B**); lean mass (**C**); energy expenditure (**D**); and oxygen consumption (**E**). The data are expressed as the mean ± SEM ($n = 4$). Results were considered statistically significant at $p < 0.05$. The differences between groups are indicated by letters, where a > b > c. C: control; HFS: high-fat and sucrose; HFS→C high-fat and sucrose, switched to the control diet; HFF: high-fat and fructose; HFS→C: high-fat and fructose, switched to control diet.

3.2. Analysis of Microbiota Composition

There is a controversy about the adverse effect of different types of simple carbohydrates in a high fat diet on the development of obesity mediated by changes in gut microbiota. The results of

the present work showed that the main phyla in the different groups studied were Bacteroidetes, Firmicutes, and Proteobacteria. The main bacterial genus modified by the consumption of HFS or HFF were *Coprococcus*, *Acidaminococcus*, and *Eubacterium*. Particularly, the HFS group showed a marked decreased in P75-a5, *Aggregatibacter*, *Bilophila*, *Sphingomonas*, *Turicibacter*, and *Klebsiella* with respect to the C group. The HFF groups showed a less pronounced effect on these genera. At the species level, a heat map was created based on the ten most modified species. There was a remarkable decrease in *C. eutactus* in the HFS and HFF groups, and there was a significant increase in *L. reuteri* and *B. fragilis* in HFF group. Particularly, *B. producta* was increased in the HFS group and *R. flavefasciens* in the HFF group (Figure 2A–D). Clustering the bacterial communities using principal component analysis (PCA) revealed that the microbiota after the consumption of HFS or HFF diets was different to that of the C group (ANOSIM R = 0.56, p = 0.001) (Figure 2E). However, when the HFS or HFF groups switched to the control diet, the gut microbiota, in part, returned to a similar extent to that of the C group. The amount of body fat was associated (r = 0.95) with the concentration of serum LPS (Figure 2F).

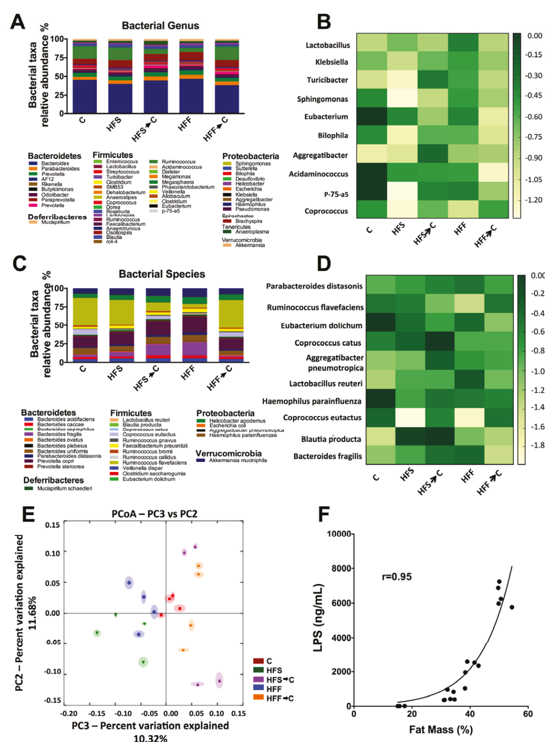

Figure 2. High-fat sucrose (HFS) or high-fat fructose (HFF) diets differentially modified the gut microbiota. Relative abundances of the gut microbiota at the bacterial genus (**A**); heat map showing the 10 most modified genera (**B**); relative abundances at the specie level (**C**); and heat map showing the ten most modified species (**D**) after the consumption of HFS or HFF diets. Unweighted principal component analysis (PCA) of gut microbiota after the consumption of different diets (**E**). The closer the spatial distance between samples the more similar they are with respect to both axes (PERMANOVA, p = 0.001). Correlation between serum LPS concentration and percent fat mass (r = 0.95) (**F**). The data are expressed as the mean ± SEM (n = 4). Results were considered statistically significant at $p < 0.05$. The differences between groups are indicated by letters, where a > b > c. C: control; HFS: high-fat and sucrose; HFS→C: high-fat and sucrose, switched to the control diet; HFF: high-fat and fructose; HFS→C: high-fat and fructose, switched to control diet.

3.3. Biochemical Parameters and Glucose Tolerance

The consumption of a high-fat diet enriched with sugars could produce biochemical alterations to different extents. We observed that the HFS group showed the highest levels of serum glucose, insulin, triglycerides, total cholesterol, and LDL cholesterol with respect to the C group ($p < 0.05$). Interestingly, the HFF group showed the same trend as the HFS group, but to a lesser extent (Figure 3). Only the HFS group showed glucose intolerance with the highest value of the area under the curve (AUC) after the glucose tolerance test. Remarkably, the HFS group showed extremely high levels of LPS (6310 ng/mL), 489-fold higher than the C group, followed by the HFF group, 192-fold higher than the C group, indicating a severe metabolic endotoxemia.

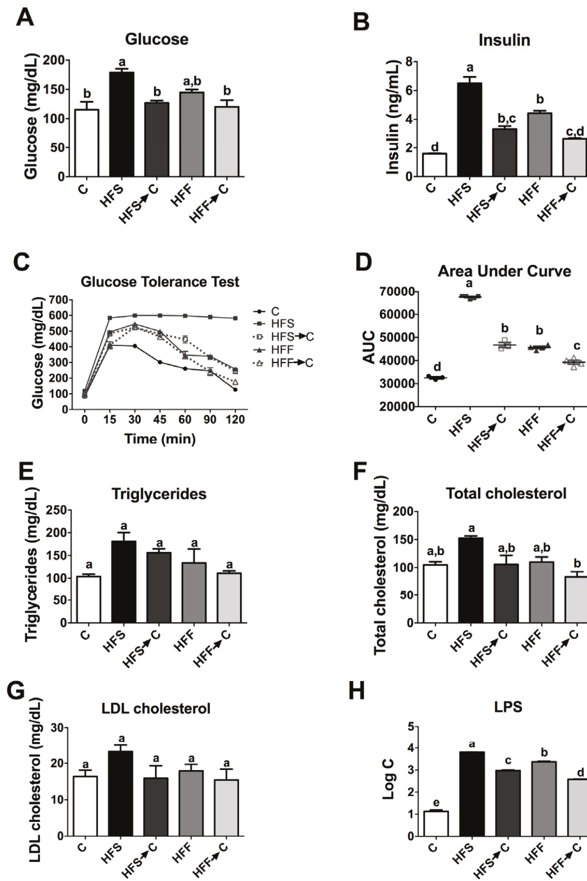

Figure 3. Serum biochemical parameters and glucose tolerance test after the consumption of different diets. Serum glucose (**A**); insulin (**B**); glucose tolerance test (**C**); area under the curve after the glucose tolerance test (**D**); triglycerides (**E**); total cholesterol (**F**); LDL cholesterol (**G**); and lipopolysaccharides (**H**). The data are expressed as the mean $\pm$ SEM (n = 4). Results were considered statistically significant at $p < 0.05$. The differences between groups are indicated by letters, where a > b > c. C: control; HFS: high-fat and sucrose; HFS→C: high-fat and sucrose, switched to control diet; HFF: high-fat and fructose; HFS→C: high-fat and fructose, switched to control diet.

3.4. Inflammation Markers and Oxidative Stress

The continuous exposure to a high-fat–high-sugar diet produce metabolic alterations in the liver, adipose tissue, and skeletal muscle, however, kidneys could also be affected by this chronic inflammatory state. As expected, the HFS group increased the inflammatory markers TLR-4, NF-κB, TNFα/β, and MCP1 compared with the C group, followed by the HFF group (Figure 4A–D, $p < 0.05$), on the contrary, we found a significant decrease in kidney UCP-1 only in the HFS group (Figure 4E). In addition, the consumption of HFS or HFF diets significantly increased ROS levels and MDA concentration in the kidney compared to the C group (Figure 4F,G). However, this effect was reverted by the consumption of the C diet. Nevertheless, the HFS group showed more oxidative damage, which was reflected in the levels of urine H_2O_2 excreted in comparison with the HFF group (Figure 4H).

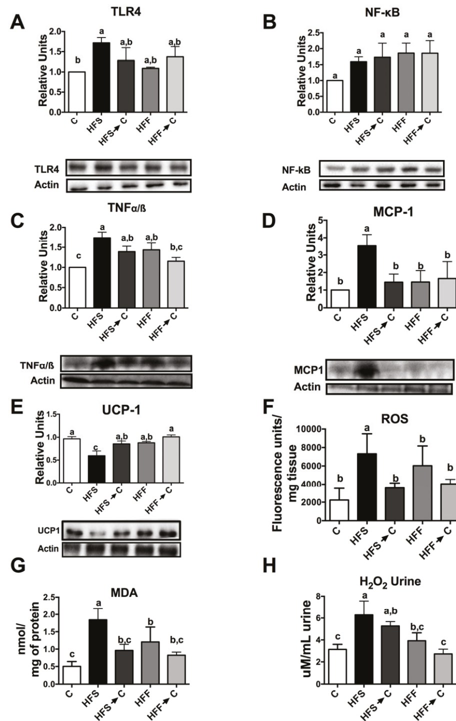

Figure 4. Relative protein abundance and oxidative markers in the kidneys of rats fed high-fat sucrose or fructose diets. Protein abundance of toll-like receptor 4 (**A**); nuclear factor kappa B (**B**); tumor necrosis factor alpha (**C**); monocyte chemoattractant protein 1 (**D**); and uncoupling protein 1 (**E**). Renal reactive oxygen species (**F**); malondialdehyde concentration (**G**); and urinary hydrogen peroxide (**H**). The data are expressed as the mean ± SEM (n = 4). Results were considered statistically significant at $p < 0.05$. The differences between groups are indicated by letters, where a > b > c. C: control; HFS: high-fat and sucrose; HFS→C: high-fat and sucrose, switched to the control diet; HFF: high-fat and fructose; HFS→C: high-fat and fructose, switched to the control diet.

3.5. Antioxidant System

Oxidative stress may be produced by an imbalance between ROS and the antioxidant system, generating inflammation. This, in turn, can produce an elevation of inflammatory cytokines. The HFS group showed the highest increase in IL-1β and IL-6 with respect to the C group (Figure 5A,B, $p < 0.05$). These results were associated with an increase in renal gene expression of NADPH oxidase, considered

as a pro-oxidant enzyme (Figure 5C). On the other hand, it has been proposed that the increase in renal lipogenesis could be responsible for the inflammation. The HFS group showed the highest expression of renal Srebp1c, a transcription factor involved in lipogenesis (Figure 5D). During this inflammation process, there was an imbalance between ROS and antioxidant enzymes, such as catalase, glutathione peroxidase (Gpx), glutathione reductase (Gr), and superoxide dismutase 1 (Sod1). The HFS group showed a significant reduction in the expression of Cat, Gpx, and Gr (Figure 5E–H), while the HFF group maintained similar levels of these enzymes as the control group, suggesting a lesser oxidative stress than the HFS group.

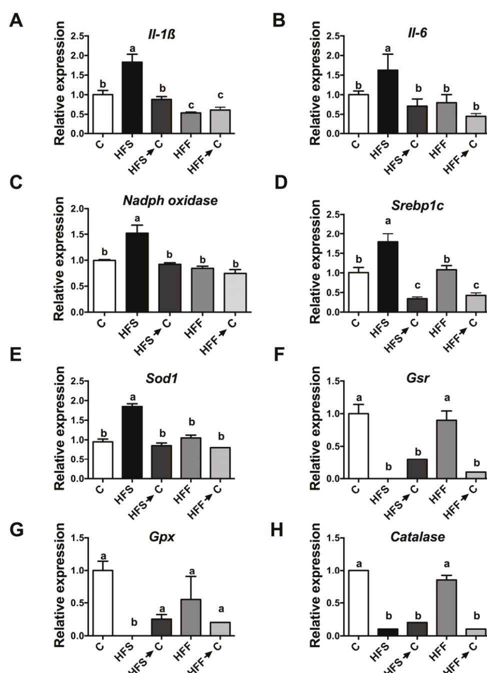

Figure 5. Relative gene expression of inflammatory cytokines, lipogenesis, oxidant, and antioxidant enzymes in kidney. Interleukin 1-β (**A**); interleukin 6 (**B**); NADPH oxidase (**C**); sterol regulatory element-binding protein-1c (**D**); superoxide dismutase 1 (**E**); glutathione reductase (**F**); glutathione peroxidase (**G**); and catalase (**H**). The data are expressed as the mean ± SEM (n = 5). Results were considered statistically significant at $p < 0.05$. The differences between groups are indicated by letters, where a > b > c. C: control; HFS: high-fat and sucrose; HFS→C: high-fat and sucrose, switched to the control diet; HFF: high-fat and fructose; HFS→C: high-fat and fructose, switched to the control diet.

4. Discussion

Obesity is a serious health problem in the world and is associated with a significant increase of all-cause mortality [25] accompanied by inflammation and frequently by disease. One of the main causes of obesity, besides physical inactivity, is the consumption of foods high in saturated fat and simple carbohydrates. Additionally, there is a debate on whether the specific type of carbohydrate consumed is responsible for the development of obesity. Increased intake of certain macronutrients, such as fat and simple carbohydrates, and particularly fructose, have been claimed to be risk factors for the development of kidney disease, hypertension, and obesity among others, due to the high consumption of high fructose corn syrup (HFCS) used in beverages [26]. However, the composition of the high fructose corn syrup is 42% fructose and 53% glucose [27], similar to the composition of

sucrose. Sucrose and HFCS deliver fructose and glucose in similar ratios to the same tissues. During digestion, sucrose is hydrolyzed to free glucose and fructose by the enzyme sucrase present in the small intestine. Then, glucose and fructose are transported into the portal circulation through the transporters SGLT-1, GLUT5, and GLUT2 on the enterocytes [28]. Whilst there is rising evidence about the deleterious effect of sucrose in the development of obesity, cardiovascular disease, and diabetes, sucrose intake is not solely responsible for obesity, but the increase in other macronutrients like saturated fat and energy [25]. Results of several animal and human studies suggest that intestinal bacteria overgrowth may be involved in pathologies, including non-alcoholic fatty liver disease, and increases in LPS binding protein that are associated with a marked increase expression of TNF-α [29]. All of this evidence suggested that the combination of a high fat-high sucrose diet is responsible, in part, for the development of obesity. The results of the present work indicated differential effects between the disaccharide sucrose and the monosaccharide fructose in combination with a high fat diet. One of the striking results of the present study was that the consumption of high fat-high sucrose or fructose diets significantly increased fat mass (51% and 40% in rats fed HFS or HFF diet, respectively). The amount of body fat was strongly associated ($r = 0.95$) with the concentration of serum LPS (Figure 2F).

These results demonstrated that obesity caused by consumption of HFS or HFF diets generated a chronic state of high-grade inflammation mediated by LPS. This state could be originated by dysbiosis in the gut microbiota (Figure 6). Animals fed HFS or HFF diet showed a significant increase in *B. producta* and a significant decrease in *C. eutactus* associated with irritable bowel syndrome [30]. The genus *Blautia* has been associated with phenylacetylglutamine, circulating metabolite derived from bacterial protein fermentation found in early renal function decline [31]. The undesirable effects of the HFF diet was possibly attenuated by the increase in *L. Reuteri* and *B. fragilis* involved in the inhibition of the growth of pathogenic bacteria [32], insulin sensitivity [33], and intestinal epithelium integrity [34]. During the development of obesity, dysbiosis in gut microbiota increased the production of LPS mainly in the HFS group, which could activate TLR4 and promote the induction of NF-κB, provoking the expression of inflammatory cytokines and ROS production. Increases in IL-1β, IL-6, and TNF-α are associated with insulin resistance and glucose intolerance [35]. This inflammatory process increased NADPH oxidase, which increased ROS formation and modified the expression of antioxidant enzymes to a different extent, however, the HFS group showed the most pronounced alteration. These modifications in the antioxidant response were associated with a redox imbalance promoting a vicious cycle in oxidative stress. Imbalance between the ROS levels and antioxidant enzymes led to the formation of MDA, which is a marker of lipid peroxidation. High levels of renal MDA were associated with kidney stress and high production of urinary H_2O_2. Interestingly, we found a significant decrease in kidney UCP-1 (Figure 4E). UCP-1 is involved in the leakage of the proton gradient preventing overproduction of mitochondrial ROS. Given that the five UCP family members are identified as able to control ROS [36], when UCP-1 decreases, the ROS control mechanism is impaired. Rats fed a HFS diet showed a significant decrease in renal UCP-1, suggesting an increase in oxidative stress. Finally, another possible mechanisms for which HFS or -F diets increase oxidative stress is by the induction of the transcription factor SREBP-1 involved in renal lipogenesis which, in turn, increases the expression of NADPH oxidase and the production of ROS. The results of the present work indicate that the combination of a high-fat diet and sucrose, and potentially fructose, can produce renal oxidative stress and a severe metabolic endotoxemia produced by a dysbiosis in the gut microbiota. Oxidative stress in the kidney does not reach kidney damage, since KIM-1, a biomarker for renal proximal tubule injury [37], was not modified (data not shown). Importantly, these deleterious effects can be partially reverted or ameliorated by the consumption of an adequate diet.

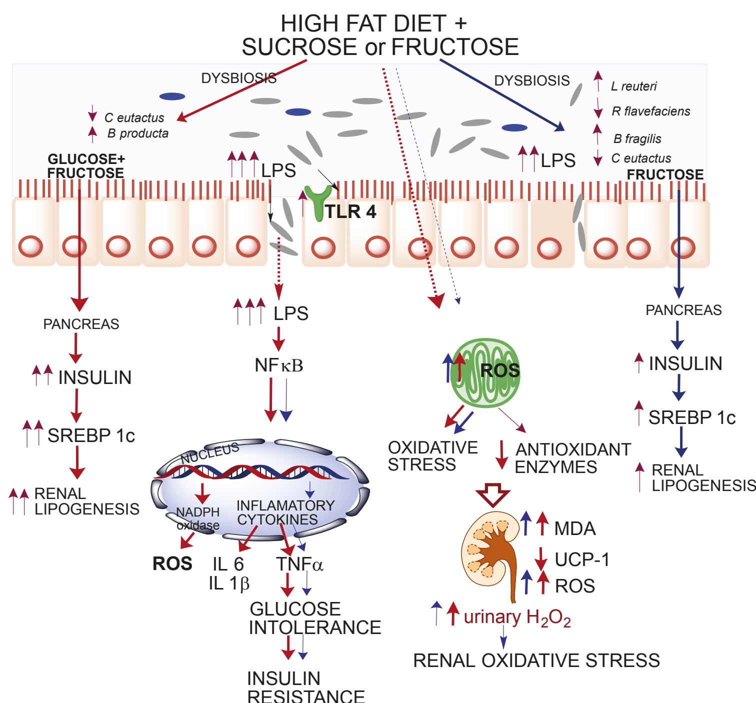

Figure 6. Graphical summary of differential effects of high fat + sucrose of high fat + fructose on gut microbiota, inflammatory cytokines, insulin resistance, and oxidative stress and lipogenesis in kidney.

5. Conclusions

The combination of a high-fat with sucrose (HFS) or fructose (HFF) diet differentially modified the gut microbiota and increased the paracellular transport of LPS generating a chronic state of high-grade inflammation. The HFS diet increased to a higher extent renal lipogenesis and inflammatory markers compared with the HFF diet. As a consequence, there was an increase in glucose intolerance and insulin resistance. Consumption of HFS or HFF diets increased the formation of reactive oxygen species (ROS) and renal oxidative stress.

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

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Review

Fructose Intake, Serum Uric Acid, and Cardiometabolic Disorders: A Critical Review

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Abstract: There is a direct relationship between fructose intake and serum levels of uric acid (UA), which is the final product of purine metabolism. Recent preclinical and clinical evidence suggests that chronic hyperuricemia is an independent risk factor for hypertension, metabolic syndrome, and cardiovascular disease. It is probably also an independent risk factor for chronic kidney disease, Type 2 diabetes, and cognitive decline. These relationships have been observed for high serum UA levels (>5.5 mg/dL in women and >6 mg/dL in men), but also for normal to high serum UA levels (5–6 mg/dL). In this regard, blood UA levels are much higher in industrialized countries than in the rest of the world. Xanthine-oxidase inhibitors can reduce UA and seem to minimize its negative effects on vascular health. Other dietary and pathophysiological factors are also related to UA production. However, the role of fructose-derived UA in the pathogenesis of cardiometabolic disorders has not yet been fully clarified. Here, we critically review recent research on the biochemistry of UA production, the relationship between fructose intake and UA production, and how this relationship is linked to cardiometabolic disorders.

Keywords: fructose; uric acid; cardiometabolic disorders; xanthine oxidase; pathophysiology; epidemiology

1. Introduction

Uric acid (UA) is the final product of purine metabolism. It is a well-known risk factor for gout [1]. Moreover, a growing body of evidence suggests that high levels of serum UA are also biomarkers for cardiovascular disease (CVD) morbidity and mortality [2].

The increased incidence of gout among rich people in the 18th and 19th centuries was principally due to high consumption of purine-containing meat. However, UA levels are rising in the 21st century too, with mean levels of >5.5 mg/dL in women and >6.0 mg/dL in men [3]. This can partly be explained by a remarkable increase in added sugars in the Western diet, especially fructose [4–6]. Accordingly, blood UA levels are higher in Western countries than in the rest of the world. In non-Western countries, hyperuricemia is relatively rare in rural communities. However, there is increased migration from rural areas to cities or communities where the Western diet is dominant and hyperuricemia is more prevalent [7].

Recent research suggests that hyperuricemia may be caused by elevated activity of the enzyme xanthine oxidase (XO) [8]. Xanthine oxidase inhibitors (XOI) have thus been proposed as a strategy for reducing UA and oxidative stress. Both are risk factors for gout, chronic kidney disease (CKD),

CVD, obesity, insulin resistance, and metabolic syndrome. Humans and great apes produce UA via XO-catalyzed oxidation of purines. Unlike other mammals, humans and great apes cannot synthesize the uricase enzyme (urate oxidase) and so cannot metabolize UA to allantoin. As a result, UA blood concentrations in humans and great apes are at least 10 times higher than in other mammals, with the consequent risk of developing hyperuricemia [9].

High UA levels favor adipose tissue formation, which was originally an evolutionary advantage for humans [7]. Nowadays, however, excess adipose tissue is considered a predisposing factor for insulin resistance, obesity, and hypertension [10]. This excessive fat storage may be due to increased consumption of fructose-enriched food and drink, which raises serum UA levels [11]. Indeed, several clinical studies have shown that the administration of allopurinol, a competitive antagonist of XO, can significantly improve endothelial function and the circulating markers of oxidative stress in patients with, or at risk of, CVD [12].

Here, we review the most relevant discoveries in the field, focusing on (i) the role of UA in cardiometabolic disorders; and (ii) the link between fructose consumption, high blood UA levels, and associated disorders, particularly CVD.

2. Search Strategy (Methods)

We conducted a literature search of different scientific databases (including Scopus, Google Scholar, PubMed, and Web of Science) for peer-reviewed studies focusing on XO, hyperuricemia, fructose, and CVD. The search strategy was designed to retrieve studies published in English from journal inception to 2016. We used an assessment framework to appraise the quality of basic research studies, prognostic studies, and methodological considerations in the analysis and publication of observational studies. The screening, study selection, and data extraction was undertaken by three independent authors. Disagreement was resolved by discussion and, if required, by a fourth independent author. We have assessed the clinical and methodological heterogeneity across the studies and, where available, included meta-analyses whenever these have been performed.

3. Purine Metabolism and Uric Acid Physiology

Purines are generated through two pathways. First, there is de novo synthesis from non-purine compounds, such as amino acids and bicarbonate, regulated by phosphoribosyl-pyrophosphate synthetase (PRPP). Second, there is the purine salvage pathway, which economizes the intracellular energy expenditure and is regulated by hypoxanthine-guanine phosphoribosyltransferase (HG-PRTase) [13]. Catabolism of purines is regulated by xanthine-oxidoreductase (XOR), coding for two distinct enzymatic forms: xanthine dehydrogenase (XDH) and XO [14]. XDH and XO catalyze the oxidation of hypoxanthine to xanthine and subsequently to UA, which is the hepatic and intestinal metabolite of purine [15,16]. The main difference between XDH and XO is that XDH-FAD reacts predominantly with NAD^+, whereas XO-FAD has higher reactivity for molecular oxygen, producing higher levels of hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-). O_2^- is then converted into oxygen and H_2O_2, either spontaneously or catalyzed by the superoxide dismutase (SOD) enzyme, (Figure 1A,B) [14]. XO is the form that is most abundant in inflamed vascular and ischemic tissues. XDH can be converted into XO via the oxidation of sulfhydryl residues or the proteolysis of XDH (Figure 1A) [17]. Allopurinol and its active metabolites oxypurinol act as competitive antagonists of XO and can lower UA levels. In addition, allopurinol has consistently been reported to prevent H_2O_2 production [12,18] (Figure 1B).

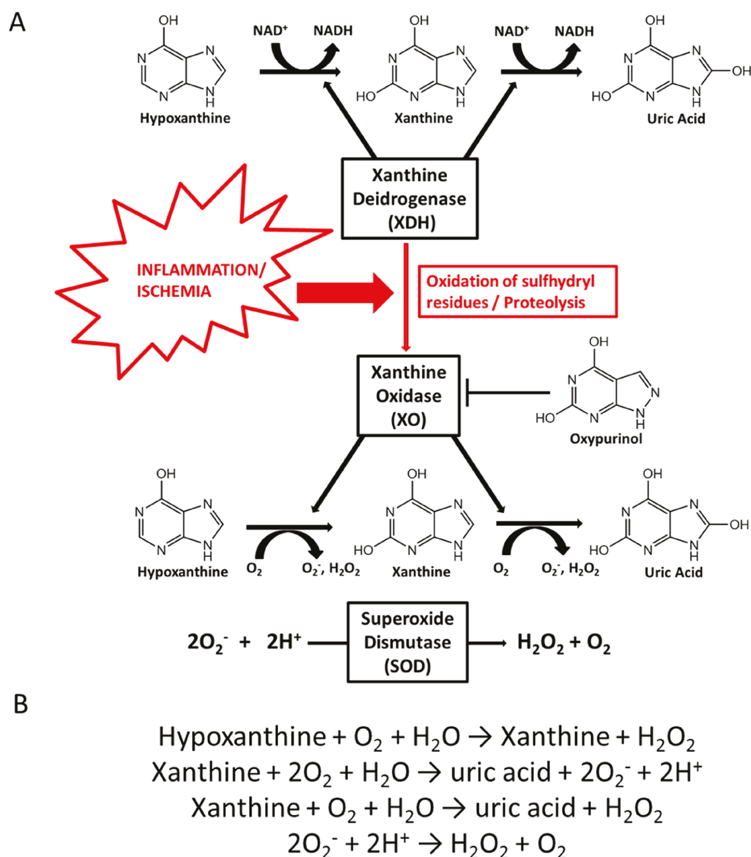

Figure 1. Uric acid formation through xanthine oxidase activity. (**A**) Under ischemic or inflammatory conditions, xanthine dehydrogenase (XDH) is converted to xanthine oxidase (XO) via the oxidation of sulfhydryl residues or proteolysis of XDH. In the presence of oxygen, XO catalyses the oxidation of hypoxanthine to xanthine and then to uric acid (UA), with consequent production of the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The competitive antagonist allopurinol is converted in the active form, oxypurinol, via XO activity, acting as an XO inhibitor; (**B**) During hypoxanthine conversion to xanthine and then UA, high levels of H_2O_2 and O_2^- are produced and converted to O_2 and H_2O_2, spontaneously or in a reaction catalyzed by the enzyme superoxide dismutase (SOD).

Most UA is filtered by the kidneys and eliminated in urine. The rest passes through the gut and is cleaved by bacteria into waste substances, which are eliminated in faeces. Inefficient renal excretion of UA is the main cause of both primary and secondary hyperuricemia [19]. Renal UA excretion is regulated by several transporters. Renal UA reabsorption is mediated by urate transporter 1 (URAT1, also known as SLC22A12) and glucose transporter 9 (GLUT9, also known as SLC2A9) [20,21]. Renal UA secretion is mediated by sodium phosphate transporter (NPT1, also known as SLC17A1) [22,23]. UA concentration can be decreased by several drugs (benzbromarone, losartan, probenecid, sulfinpyrazon). These drugs mostly work by URAT1 inhibition, which explains their uricosuric effect [24]. UA excretion is also regulated by breast cancer resistance protein (BCRP, also known as ABCG2), which belongs to the superfamily of ATP-binding cassette (ABC) transporters [25–27]. Unlike ABC transporters, however, BCRP has only one N-terminal ATP-binding

domain [28–30]. Reduced intestinal excretion of UA is often associated with polymorphisms of the BCRP gene [26] or with a lack of BCRP dimerization, which is due to oxidative stress [31,32].

As noted above, most mammals can degrade UA to allantoin in a reaction catalyzed by uricase, an enzyme present in peroxisomes. Allantoin is subsequently degraded to urea for excretion [33,34]. Most mammals thus have relatively low UA circulation levels (0.5 to 2.0 mg/dL). However, humans and great apes cannot synthesize functional uricase and therefore have much higher blood UA levels.

According to Neel's hypothesis, our closest evolutionary ancestors underwent functional genetic mutations, which silenced genes involved in the degradation of UA and the synthesis of vitamin C [10], which is the major water-soluble intracellular free-radical scavenger in human plasma [35]. These mutations increased de novo lipogenesis and weight gain [36]. This is because vitamin C competes with UA for renal resorption in the proximal tubule [37–39], producing a uricosuric effect. UA causes endoplasmic reticulum stress; this, in turn, activates SREBP-1c, which stimulates fat accumulation in the liver [40]. Indeed, Cheung et al. recently reported that XOR-knockout mice have a central defect in adipogenesis and fail to gain fat [41–43]. Thus, there is a positive correlation between circulating UA and obesity, especially visceral obesity [44]. Accordingly, although hyperuricemia is often considered to be a secondary phenomenon in metabolic syndrome, it is also an independent predictive factor for obesity and hyperinsulinemia [4]. In themselves, UA accumulation and lack of vitamin C do not cause obesity. Rather, they increase susceptibility to obesity and diabetes as a result of an interaction between genetic factors (mostly a polygenic contribution) and environmental factors such as lifestyle, social influences, and fetal surroundings [11,45].

4. Fructose Metabolism and the Mechanisms by Which Fructose May Contribute to Uric Acid Production

In the past 100 years, there has been a progressive rise in blood UA levels, especially in Western countries. This is associated, at least in part, with a rise in the number of people consuming a Western diet. In particular, there has been increased consumption of fructose-containing sugars [46,47], sucrose, high-fructose corn syrup, soft drinks, and fruit juices [11,48].

Fructose is present as a monosaccharide in fruits and vegetables [49], as a disaccharide in sucrose (with D-glucose), and as oligo- and polysaccharides (fructans) in many plants. It is also used as an added sweetener for food and drink, and as an excipient in pharmaceutical preparations, syrups, and solutions [50]. Following fructose ingestion, blood glucose levels are lower (GI 32) than after ingesting a similar amount of glucose (GI 100) or sucrose (GI 68). Similarly, insulin levels do not increase significantly after fructose ingestion. Moreover, fructose has a greater sweetening power than sucrose, so smaller quantities of fructose can be used to sweeten foods. Finally, its calorific value is 3.75 kcal/g, slightly lower than that of sucrose (3.92 kcal/g) [51]. It is not yet clear if a moderate intake of fructose significantly promotes the formation of advanced glycation products (AGE), which damage tissues and thus contribute to ageing and metabolic disorders [52]. Several studies suggest that a fructose-rich diet has negative metabolic consequences, including AGE formation [52–54]. However, the effect of a long-term fructose intake on AGE accumulation in tissues has not yet been studied in healthy volunteers.

Hyperuricemia is caused by the overproduction and/or underexcretion of UA. It has been reported that metabolism of fructose stimulates UA production, since transient ATP depletion commonly occurs with the generation of AMP [55] and reduced UA excretion [56]. Briefly, during fructose metabolism, fructose is phosphorylated into fructose 1-phosphate in a reaction catalyzed by fructokinase primarily in the liver. This reaction is rapid, has no negative feedback, and hugely decreases the levels of intracellular phosphate and ATP [57]. Next, the enzyme fructose-1-p aldolase (also known as aldolase B) breaks fructose 1-phosphate into dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde. When there is a high intake of fructose, phosphorylation into fructose 1-phosphate is fast, but the reaction with aldolase is slow (Figure 2). Hence, fructose 1-phosphate accumulates, and intracellular phosphate decreases. This decrease stimulates AMP deaminase (AMPD), which catalyzes

the degradation of AMP to inosine monophosphate, increasing the rate of purine degradation [58] (Figure 2). The purine degradation produces UA [59] and generates mitochondrial oxidants [36] (Figure 2). Mitochondrial oxidative stress then induces aconitase inhibition in the Krebs cycle, with accumulation of citrate and stimulation of ATP citrate lyase and fatty acid synthase. The result is de novo lipogenesis and hepatic fat accumulation [36].

Physiologically, the increase in intracellular UA is followed by an acute rise in circulating levels of UA, which is likely due to its release from the liver [57,60]. Fructose also stimulates UA synthesis from amino acid precursors such as glycine [61]. Moreover, long-term fructose administration suppresses renal excretion of UA, resulting in elevated serum UA levels [19]. Kaneko and colleagues found that a single administration of fructose affects the excretion of UA to the intestinal lumen, inducing the suppression of BCRP dimerization by reactive oxygen species (ROS)-derived production of dinucleotide phosphate (NADPH) oxidase (NOX) [56].

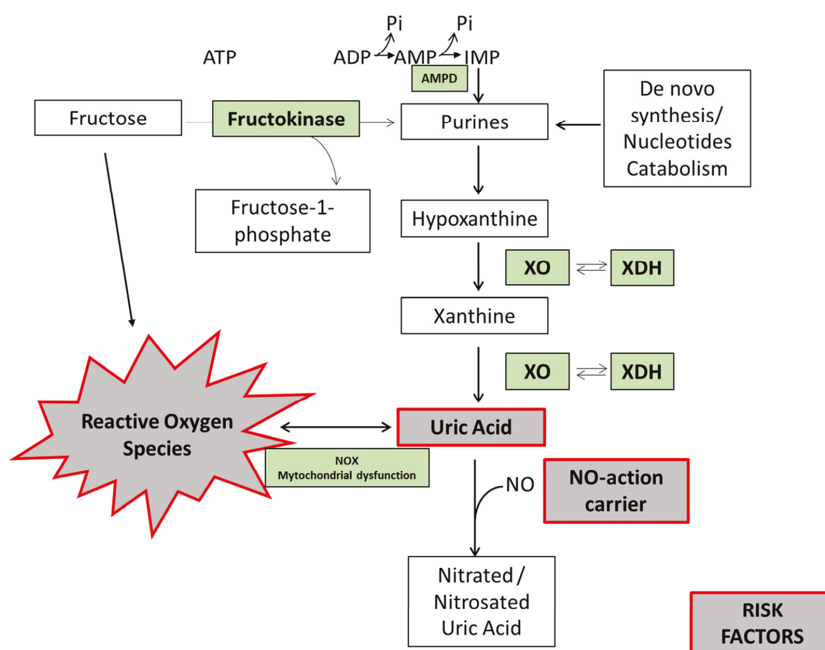

Figure 2. Fructose-induced uric acid formation: risk factor biomarkers. In hepatocytes, fructokinase catalyzes the rapid phosphorylation of fructose to fructose-1-phosphate, using ATP as a phosphate donor. Intracellular phosphate (Pi) levels decrease, stimulating the activity of AMP deaminase (AMPD). AMPD converts AMP to inosine monophosphate (IMP). IMP is metabolized to inosine, which is further degraded to xanthine and hypoxanthine by xanthine oxidase (XO), ultimately generating uric acid (UA). UA can react with nitric oxide (NO), reducing NO bioavailability and inducing dinucleotide phosphate oxidase (NOX) activation and mitochondrial dysfunction. In turn, this promotes oxidative stress and endothelial dysfunction. Fructose per se can also induce oxidative stress.

5. Mechanisms by Which Fructose-Uric Acid May Contribute to Cardiometabolic Disorders

UA has been described as a "paradox molecule" with opposing roles. At physiological concentrations (much higher than ascorbate concentrations in plasma), it is a powerful oxygen radical scavenger in extracellular hydrophilic environments such as human plasma. It may protect the erythrocyte membrane from lipid peroxidation [35]. UA can react with O_2^-, H_2O_2, hydroxyl radical

(OH^-), and particularly peroxynitrite ($OONO^-$) [35,62,63]. However, several authors suggest that UA also acts as a prooxidant inside the cell under certain inflammatory conditions, such as atheromatous plaque formation [35,64]. Recent studies have shown that UA can induce intracellular oxidative stress and proinflammatory effects in various cell types [65,66]. It does this by stimulating NOX [67,68] and by altering mitochondrial function with the consequent alteration in fat synthesis [36]. Fructose also induces NOX activation [69,70].

Oxidative stress significantly contributes to the development of insulin resistance and imbalance in vascular homeostasis, including endothelial cell dysfunction, atherosclerosis, vascular calcification, and impaired myocardial energetics, stimulating the production of interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) [71,72]. Heme oxygenase 1 (HO1), a potent antioxidant, decreases UA levels and adipocyte dysfunction by decreasing levels of ROS and XO [73]. XO is one of the major endothelial sources of O_2^- and H_2O_2. XO levels are substantially elevated in patients with coronary disease or carotid stenosis, and there is an inverse relationship between XO levels and endothelium-dependent vasodilation [74]. Accordingly, UA inhibits the bioavailability of nitric oxide (NO), which is a vasodilator [65,75]. Extracellular UA can also enter endothelial cells and vascular smooth muscle cells through URAT1, GLUT9, and potentially other transporters [76,77] activating the NF-κB axis, which leads to an increase in MCP-1, IL-8, VCAM-1, and ICAM-1 [78]. As a result, it has been hypothesized that intracellular XO activity and increased ROS production might be factors in endothelial dysfunction, which leads to the development of essential hypertension [79]. In this regard, ROS generation and vascular endothelial dysfunction can be reduced by drugs such as allopurinol and febuxostat, which inhibit XO activity and consequently reduce UA production [80].

$OONO^-$ is a potent non-radical oxidant species formed by the reaction between NO and O_2^-, which commonly occurs in the vascular endothelium [81]. NO is usually generated by NO synthase (NOS), However, under hypoxic conditions, XO can also modulate NO concentrations [82,83]. Since reducing NO bioavailability induces endothelial dysfunction and oxidative stress, XO inhibition could prevent oxidative damage, at least in part, by restoring NO generation [84,85]. Several studies have shown that, under aerobic conditions, UA can react with NO but not with $OONO^-$, producing a nitrosated derivative [35,86,87]. Therefore, despite the general belief that survival is associated with increased antioxidant capacity, the opposite appears to be true: the ability to increase oxidative stress may have been associated with survival among early hominoids.

6. Evidence from Clinical Studies, Relevance for Humans

Physiologically, circulating UA levels increase with age. UA levels are lower in women of childbearing age, rising after menopause to levels similar to those in men [24]. There is still no clear threshold, above which uricemia becomes "abnormal". The pathophysiological approach uses the supersaturation concentration of UA, 6.8 mg/dL at 37 °C, as a cut-off value, as indicated by guidelines for gout management [88]. However, recent findings suggest that this should be revised with reference to rheumatic, cardiovascular, and renal risk. Hyperuricemia is increasing in prevalence, as are its associated pathological conditions, such as metabolic syndrome, CKD, and CVD [8,89]. The average serum UA levels in the general population are rising [90] due to dietary changes, rising body mass indexes (BMI), and improved life expectancy.

Several in vitro and in vivo studies have shown that high fructose consumption increases blood UA levels [4,55,56,91]. In addition, consuming fructose over several days [70] and intravenous fructose administration [92] are both associated with an increase in fasting serum UA levels. It is not yet clear whether circulating UA levels increase when fructose is taken in small doses over several days or when it is taken as a single large dose. It is also unclear whether a high-fructose diet (HFrD) alters the renal clearance of UA or the fractional excretion of UA (UAFE) [93].

A relatively old meta-analysis of 21 controlled feeding trials of at least seven days showed that isocaloric exchange of fructose for other carbohydrates did not affect serum UA. However, in nondiabetic participants, hypercaloric supplementation of control diets with fructose (+35% excess

energy) at extreme doses (213–219 g/day) significantly increased serum UA compared with the control diets alone (MD = 31.0 mmol/L (95% CI: 15.4, 46.5)) [94]. This meta-analysis was conducted before the publication of more recent trials, which confirmed the relationship between fructose intake and serum UA levels [95–97]. Thus, the available evidence is yet partly conflicting.

Notably, the fructose-induced increase in serum UA may cause acute damage (post-assumption). By excluding shorter studies, the meta-analysis may have excluded insights into this issue [97]. Finally, a fructose-restricted diet has been associated with a decrease in serum UA [98].

Lecoultre and colleagues performed a study on healthy subjects with two approaches: four to six days on an isoenergetic low-fructose diet (LFrD) and then either 6 days on a hyperenergetic HFrD with 34% excess energy as fructose or four days on a weight-maintenance HFrD in which 30% starch was substituted with fructose [42]. There was no difference in urinary UA excretion after the HFrD and the LFrD [42]. This suggests that UA production increases mainly when there is abnormally high hepatic metabolism of fructose. These findings also demonstrate that decreased urinary UA excretion may contribute to fructose-induced hyperuricemia. This mechanism could substantially increase the risk of gout in people who consume high amounts of fructose. Moreover, chronic exposure to fructose favours the onset of metabolic syndrome and increases insulin resistance [51].

Many experiments have shown that hyperuricemia may have a potential role in endothelial dysfunction and reduced NO bioavailability. However, several other studies have failed to demonstrate that an increase in blood UA levels can contribute to the onset of coronary and cardiovascular diseases. These studies also found no association between raised serum UA levels and the incidence of cardiovascular events [35,99–103]. It is not yet possible to say whether UA is a causal, compensatory, or coincidental factor for CVD [104]. Generally, gout and hyperuricemia patients also suffer from hypertension, CKD, insulin resistance, and obesity [105]. However, no clear link has yet been demonstrated between a HFrD and CVD. A recent systematic review and meta-analysis of prospective cohort studies was undertaken to quantify the association between intake of fructose-containing sugar (high-fructose corn syrup, sucrose, and fructose) and incidence of hypertension. This review searched MEDLINE, EMBASE, CINAHL, and the Cochrane Library for relevant studies [106]. It demonstrated that there was no association between a high total fructose intake and an increased risk of hypertension in three large prospective cohorts of men and women in the USA. In contrast, the same group conducted another systematic review and meta-analysis of prospective cohort studies to quantify the association between the consumption of fructose-containing sugar-sweetened beverages (SSBs) and the risk of hypertension. This demonstrated that SSBs were associated with a modest risk of developing hypertension in six cohorts [107].

The well-characterized Mediterranean cohort of the Brisighella Heart Study has a high intake of fruit [108]. Here, the increase in blood UA levels in the general population has been associated with increased incidence of hypertension and diabetes [109], LDL oxidation [110], arterial stiffness [111], impaired cognitive function [112], and heart failure [113].

In general, epidemiologists and other scientists have attempted to prove a causal link between dietary fructose intake and metabolic disorders, such as obesity, diabetes, and metabolic syndrome. However, while some studies have found a link, other studies have not [114].

Recent relevant clinical research has sought to verify if XO inhibition could benefit patients with high circulating UA levels, focusing on the XO inhibitor allopurinol and its active metabolite oxypurinol. Allopurinol has displayed beneficial effects on blood pressure in adolescents with newly diagnosed essential hypertension [89], and in patients with heart failure, coronary artery disease, and stroke [115]. Allopurinol may thus improve endothelial function and endothelium-dependent vasodilation in chronic heart failure patients [18].

XO inhibition has also been shown to improve a range of surrogate markers in hyperuricemic patients with CVD [9]. Wu and colleagues have demonstrated that high blood UA levels could be an independent predictor of mortality in patients with severe heart failure [116]. Baldus and colleagues have shown that oxypurinol improves myocardial contractility in patients with ischemic

cardiomyopathy [117]. It also improves coronary vascular endothelial dysfunction in patients with coronary artery disease (CAD) [74]. The same group has shown that, without XO inhibition, lowering UA by uricosuric treatment (with benzbromarone) has no beneficial effect for chronic heart failure (CHF) patients [118]. This finding suggests that it is upregulated XO activity, rather than increased UA, that is actively involved in hemodynamic impairment in CHF. However, other studies suggest that elevated serum UA levels may be a risk marker for developing CVD [119,120]. Therefore, the scientific community needs to clarify the role of XO activity in CVD, so that the administration of natural or synthetic XO inhibitors as therapeutic agents for CVD can be properly evaluated [121,122].

7. Conclusions

In summary, a specific causal link between fructose consumption, hyperuricemia, and CVD has not yet been established. There is an association between UA and established cardiovascular risk factors, and there is a limit to how much population-based studies can adjust for confounding variables. As such, it is not yet possible to conclude that fructose intake is the main contributor to an increase in blood UA, and that this detrimentally affects vascular health. Further studies are required to prove or exclude a causal correlation between dietary fructose intake, UA production, and metabolic disorders. This would allow researchers to better understand which patients would obtain the greatest preventative benefit from reducing their UA levels, with diet and/or with XO inhibitors.

An important issue to consider is the bioavailability of XO inhibitors, especially the ability to cross cell plasma membranes. To address this, members of our group recently developed a cell-based biosensor that only measures intracellular XO activity and its inhibition by drugs that cross cell membranes [123]. The scientific community will need to focus on bioanalytical methods for directly monitoring XO activity and UA production. These will be useful tools for predicting the potential effects of new XO inhibitors, which could be used to treat hyperuricemia linked with cardiometabolic disorders.

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nutrients

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Review

Fructose Consumption in the Development of Obesity and the Effects of Different Protocols of Physical Exercise on the Hepatic Metabolism

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Abstract: Fructose consumption has been growing exponentially and, concomitant with this, the increase in the incidence of obesity and associated complications has followed the same behavior. Studies indicate that fructose may be a carbohydrate with greater obesogenic potential than other sugars. In this context, the liver seems to be a key organ for understanding the deleterious health effects promoted by fructose consumption. Fructose promotes complications in glucose metabolism, accumulation of triacylglycerol in the hepatocytes, and alterations in the lipid profile, which, associated with an inflammatory response and alterations in the redox state, will imply a systemic picture of insulin resistance. However, physical exercise has been indicated for the treatment of several chronic diseases. In this review, we show how each exercise protocol (aerobic, strength, or a combination of both) promote improvements in the obesogenic state created by fructose consumption as an improvement in the serum and liver lipid profile (high-density lipoprotein (HDL) increase and decrease triglyceride (TG) and low-density lipoprotein (LDL) levels) and a reduction of markers of inflammation caused by an excess of fructose. Therefore, it is concluded that the practice of aerobic physical exercise, strength training, or a combination of both is essential for attenuating the complications developed by the consumption of fructose.

Keywords: fructose; obesity; liver; aerobic exercise; strength exercise; combined exercise

1. New Story/Old Enemy

The high consumption of sugary beverages rich in fructose is directly related to the development of obesity and its consequences, such as metabolic syndrome [1–3]. Concomitant with the increased incidence and prevalence of obesity and metabolic syndrome, the consumption of fructose has increased around 30% in the last 40 years [4]. More specifically, because fructose is less able to promote satiety and is more palatable, it will stimulate a higher consumption of food [4], and alter the metabolism of lipids and carbohydrates, thereby favoring the synthesis and accumulation of fat [5].

The accumulation of adipose tissue has come to be considered a global public health problem. The hypertrophy of this tissue generates harmful effects on the organism through the secretion of various types of adipokines, and for this reason obesity happens to be considered one of the major risk factors for the development of metabolic syndrome and, consequently, is listed as one of the most serious problems in relation to quality of life [6]. According to epidemiological data, it is expected that by 2025, approximately 18% of men and 21% of women worldwide will be considered obese [7]. With that in mind, because fructose consumption is strongly associated with the development of obesity, studies aimed at evaluating its role in the development of obesity are of paramount importance for a better understanding of the development process of obesity.

A recent meta-analysis found that consumption of fructose-rich beverages leads to increased body weight gain, elevated systolic blood pressure, hyperglycaemia, hyperinsulinaemia, and increased serum triglyceride (TG) concentrations [8]. On the other hand, it was demonstrated that the replacement of fructose by glucose in beverages for 4 weeks resulted in an improvement in insulin sensitivity in adipose tissue in young subjects diagnosed with non-alcoholic fatty liver disease (NAFLD) [9]. The harmful effects of fructose can also be found from the first months of life. Newborn babies, who were breastfed by mothers who had ingested this sugar during pregnancy or lactation, presented metabolic alterations that may last throughout life. Zheng and collaborators [10] showed that children of mothers who consumed fructose had increased body weight, food intake, and circulating levels of leptin, and decreased insulin sensitivity. Later Hu and collaborators [11] demonstrated that each glass or can of fructose-enriched beverage ingested daily by a child increases by up to six times the probability of that child becoming obese during adulthood [12], thereby listing fructose as an important sugar in the genesis of obesity. Therefore, this article aimed to review the current literature to clarify how high fructose consumption may lead to metabolic diseases in rodents and humans, and how different types of physical exercise might be important to attenuate those complications.

2. Methodology

The present study is characterized as a narrative literature review. The articles used in this review were searched in the electronic database PubMed (Medline), using the following descriptors and their combinations: fructose, high fructose, obesity, aerobic exercise, endurance exercise, aerobic training, endurance training, strength exercise, resistance exercise, strength training, resistance training, combined exercise, concurrent exercise, combined training, and concurrent exercise. Moreover, to select the articles related to the aim of this review, the title and summary of searched publications were read [13]. When the articles matched to the objectives of this review, they were fully read. The research was conducted in November 2016, and even though the authors did not set limits for the year of publication, the most recent articles about this issue were preferred. Furthermore, only full text articles were used in this review.

3. High Fructose Intake and Its Consequences on Metabolic Health

3.1. Animal Evidence

As previously discussed, the rates of obesity and fructose consumption are following the same pattern; thus, several studies were conducted to understand how fructose consumption was related to weight gain. In an experiment in which rodents were fed fructose or sucrose during 8 weeks, the authors observed that even though there were no differences between the total caloric intake, animals fed fructose gained more weight than those fed sucrose [14]. In the same study, when the intervention period lasted 6 months, male and female rodents fed fructose had an accumulation of adipose tissue mainly in the abdominal area and an increase of serum TG levels.

Moreover, several studies corroborated with the evidence that high fructose consumption might lead to accumulation of adipose tissue, systemic inflammation, obesity, oxidative stress, and consequently insulin resistance in different tissues [15–18]. Furthermore, mice who received fructose

in their water had an elevation of serum proteins with proinflammatory activity, such as interleukin 1β, interleukin-6, and tumor necrosis factor alpha (TNF-α) [15]. Rodrigues and colleagues [17] demonstrated that the replacement of sucrose for fructose in one meal was enough to establish inflammation in liver and in adipose tissue. In addition, one hour after intervention, TNF-α levels of those fructose fed animals were higher than the sucrose fed animals, and this raise remained for at least 4 h. A similar behavior was observed with interleukin-6 in adipose tissue. Another study demonstrated the antioxidant system impairment of animals fed a diet composed by 60% of fructose, where those animals presented increased malondialdehyde levels [16]. Moreover, the authors further demonstrated that both fasting and homeostasis model assessment-estimated insulin resistance (HOMA-IR) were elevated after 8 weeks of treatment with high fructose diet, indicating severe insulin resistance [16].

Furthermore, high fructose consumption may contribute to the development of obesity and metabolic complications, since it affects the central nervous system and might disturb hunger and satiety control. Rodents hydrated with fructose solution showed higher levels of ghrelin than animals hydrated with solutions of sucrose or glucose [19]. Moreover, the anorexigenic neuropeptides expression was reduced in all groups treated with carbohydrates, but only the group that received fructose solution presented an elevation in cannabinoid 1 (CB1) receptor messenger RNA (mRNA) levels. In addition, Huang and colleagues [20] observed that after an 8-week intervention, the leptin levels were approximately 100% higher in animals fed high fructose diet than those fed chow diet.

On the other hand, liver seems to be a key organ in the development of metabolic complications related to fructose-rich diets [21–24]. Wistar rats were fed diets containing 65% of the calories from fructose, and the accumulation of fat and collagen in the liver tissue was as evident as observed in animals fed a diet rich in saturated fat, promoting apoptosis in this tissue and activation of proteins related to endoplasmic reticulum stress and inflammatory process [21]. Recently, an important study with primates showed that diets rich in fructose were able to induce a hepatic steatosis stage, with lipid droplet size positively correlated to time of exposure to the diet [24]. Moreover, they observed that high fructose consumption might induce liver damage even without excessive intake of fat or calories.

3.2. Human Evidence

Given the robustness of the evidence presented by basic research on the deleterious effects of high fructose consumption on metabolic parameters, it is expected that excessive fructose intake might lead to metabolic complications in humans. In 2010, Goran and colleagues [25] demonstrated that countries which used fructose-rich sweeteners had a higher incidence of diabetes, even though the incidence of obesity and the total amount of sugars ingested did not increase. Recently, Lin and colleagues [26] carried out a study including 1454 adolescents, in which they observed that high fructose consumption was related to higher levels of fasting insulin, serum uric acid, and central adiposity.

Furthermore, some studies in humans have also shown an increase in circulating TG levels due to high fructose consumption [27,28]. Silbernagel and colleagues demonstrated that after consuming 150 g of fructose daily for 4 weeks, subjects presented an elevation of 350 mg/L of serum TG levels, while for participants who consumed the same amount of glucose, their TG levels remained unchanged [27]. In addition, Abdel-Sayed and colleagues [28] observed that TG levels may increase earlier, with only 7 days of fructose supplementation. These authors also demonstrated that high fructose diet increased lactate production and hampered mobilization and lipid oxidation. Similar results were found by Lê and colleagues [29] in a study where healthy subjects increased very low density lipoprotein (VLDL) level after one week consuming fructose solutions thrice daily. Moreover, leptin levels of these subjects were also increased after one week of intervention, reaching a 48% raise after four weeks of experimentation. On the other hand, healthy women did not present changes in any parameters related to hunger and satiety control, such as insulin, leptin, and ghrelin levels after a fructose-rich meal [30]. Therefore, more studies are needed to better understand how variables such as dietary exposure time, fructose concentration, and number of daily meals may influence the level of hormones related to hunger and satiety control in humans.

Moreover, liver is one of the main targets of the harmful effects of high fructose consumption. Schwarz and colleagues [31], using magnetic resonance spectroscopy, observed that 9 days of fructose-rich diet was enough to raise liver lipids accumulation with a significant increase in postprandial de novo lipogenesis and complications in the control of hepatic glucose production. Sobrecases and colleagues [32] found similar results in their study with healthy men who received isocaloric solutions of fructose or saturated fat for 7 days. At the end of the intervention period, the intrahepatocellular lipids accumulation was similar between the groups. On the other hand, only the fructose solution group increased VLDL levels. Thus, the authors concluded that a short-term intervention with fructose-rich solution might promote fat liver accumulation and contribute to the development of insulin resistance in this tissue.

4. The History of Fructose Consumption

Sucrose has been widely used since the Middle Ages as a dietary component. It was originally derived from sugar cane in countries such as New Guinea and the Indian subcontinent, from where it was transported to Europe where it was consumed only by royalty and the most fortunate. In the fifteenth century, the countries of the Iberian Peninsula began to increase the planting of sugar cane and sugar production. However, only after the 1500s, with the discovery of the Americas and the use of slave labor, did cane planting and sugar export begin to expand. Consequently, with the increase in its production, sugar began to be consumed by the whole population, becoming widely used for the production of sweets during the eighteenth century, so that the average consumption per capita of sugar in England jumped from 1.8 km in the year 1700 to 8.1 km in 1800 [4]. Finally, it was only in the 1960s that fructose was included as a sweetener in the diet with the production of "sweet corn-based syrups" known as "high-fructose corn syrups" (HFCSs) [33].

The inclusion of HFCS as a sweetener brought benefits such as longer shelf life and lower cost [4]. Thus, the creation of HFCS-42 in 1967 and HFCS-55 in 1977 (HFCS-42 consisted of 42% and HFCS-55 consisted of 55% fructose, respectively) promoted new opportunities for the sweetener and beverage industries. Since then, consumption of sucrose and HFCS has grown exponentially. In the 1970s, syrup accounted for less than 1% of the calories ingested through caloric sweeteners in the United States, reaching a rate of 42% in the 2000s and it is currently found in most foods containing caloric sweeteners [34]. While efforts to combat the development and treatment of obesity are rising, food production containing fructose, sucrose, or HCFs is increasing quickly. Currently, an American individual consumes, on average, 72 g/day of sugar, corresponding to approximately 275 kcal/day [35]. Over the years, syrups rich in fructose have been produced from a variety of other raw materials, such as sugar cane, tapioca, rice, wheat, manioc, and beet [33]. This led several research groups to identify the intake of this nutrient as the main engine of the current obesity pandemic [36,37].

5. Sweet Poison

Found in several processed foods, fructose is usually either bonded to glucose molecules (sucrose) or not (HFCS). After ingestion, the fructose molecules pass through the digestive tract and reach the small intestine where they are rapidly absorbed by the intestinal epithelium through the glucose transporters (GLUT5) [38] and then released into the bloodstream. In the bloodstream, this nutrient is absorbed by different tissues, but mainly by the liver, which has high amounts of glucose transporter 2 (GLUT2) [38]. On the other hand, virtually no fructose is absorbed by pancreatic beta cells, because they lack expressive amounts of GLUT2 and GLUT5 transporters [39]. This characteristic is extremely important for understanding the pathogenesis of obesity. While glucose triggers the release of insulin by pancreatic beta cells, fructose is not able to do so [35]. In addition, this nutrient also appears to not stimulate leptin release and does not suppress the release of ghrelin in starvation [35,40]. These three peptide hormones play a fundamental role in the control of food intake and basal energy expenditure, acting both in the central nervous system and peripheral tissues [41,42]. While ghrelin increases the forkhead box protein 01 (FoxO1) binding to deoxyribonucleic acid (DNA), both insulin

and leptin phosphorylate FoxO1 release it from DNA, thereby reducing the hunger signal and hepatic gluconeogenesis and contributing to increased energy expenditure [43,44]. Animal studies have shown that following the administration of fructose directly into the hypothalamus, rodents showed increased food intake, while glucose injection had the opposite effect [45]. These findings explain in part the increased prevalence of obesity in individuals who consume this nutrient in the form of sugary drinks or industrialized foods [11]. Although the lack of effects on satiety, energy expenditure, and glucose uptake in itself is extremely damaging, fructose also activates extremely harmful signaling pathways in liver tissue cells.

Most cells have reduced GLUT2 content, which leads to a marked transport of this nutrient to the hepatocytes where the presence of these transporters is abundant [38]. Inside the cytoplasm, fructose may provide an energetic substrate for hepatic glucose production (gluconeogenesis) or be rapidly phosphorylated and converted to fructose 1-phosphate (fructose 1-P) by the action of the enzyme fructokinase, which uses the energy of an adenosine triphosphate (ATP) molecule. This conversion decreases energetic availability in the hepatocyte and increases the contents of intracellular adenosine diphosphate (ADP) and adenosine monophosphate (AMP). Elevated levels of ADP and AMP activate mitochondrial energetic pathways, increasing the NAD^+/NADH (nicotinamide adenine dinucleotide) ratio. An increased NAD^+/NADH ratio leads to increased activity of Sirtuin-1 (SIRT-1) and phosphoenolpyruvate carboxykinase (PEPCK) [46]. Finally, the strong deacetylation activity of SIRT-1 [25] deacetylates the already known FoxO1 protein, increasing its binding to nuclear DNA and triggering the expression of the protein kinase C (PKC) and peroxisome proliferator-activated receptor-gama coactivator 1 alpha (PGC-1α) genes [47,48]. All this fine mechanism triggered by the simple elevation of fructose in the intracellular environment results in increased rates of hepatic gluconeogenesis and hyperglycaemia. In addition to the effects on the control of glycaemic homeostasis, increased AMP concentration triggered by fructose activates the AMP deaminase enzyme, starting the hypoxanthine pathways, which increases the inflammatory process and produces uric acid. Uric acid is a potent inhibitor of the nitric oxide synthase enzyme that acts on the production of nitric oxide by the conversion of arginine to citrulline [49]. Nitric oxide plays a key role in endothelial relaxation leading to vasodilation through increased lumen diameter of the arteries. As a result, fructose, through increased levels of uric acid, may prevent the proper functioning of blood vessel gauge control pathways and contribute to elevated systemic blood pressure [50]. All these mechanisms initially triggered by the transport of fructose into the hepatocyte are extremely relevant and contribute to the development of diseases such as hyperglycaemia, gout, endothelial inflammation, and arterial hypertension [2,51].

Raising the levels of fructose-1P inside cells activates other important energy pathways. The 1P form of this nutrient activates peroxisome proliferator-activated receptor-gama coactivator 1 beta (PGC1-β) protein, which in turn increases the expression of the sterol regulatory element-binding protein 1c (SREBP1c). SREBP1c initiates the transcription of fatty acyl-coA synthase (FAS) and acetyl-CoA carboxylase (ACC) proteins [52]. All these mechanisms prepare the cell for an increase in fatty acid synthesis using the carbon chains supplied by intracellular fructose. The fructose-1P is converted into glyceraldehyde and dihydroxyetonephosphate, two intermediates of glycolysis. This process, called "fructolysis", requires the activity of the enzyme fructose-1P aldalose [53]. Glyceraldehyde provides carbon chains for the production of pyruvate, which goes to the mitochondria, where it is reduced to Acetyl-CoA. In the mitochondrial matrix, Acetyl-Coa is converted to citrate through the Krebs cycle and then migrates from the mitochondria to cytoplasm, where it will be converted into malonyl-CoA by the enzyme ACC. The excess of malonyl-coA in cytoplasm inhibits the activity of the protein carnitine palmitoyl transferase 1 (CPT-1), thereby blocking the transport of lipids to the mitochondria, and stopping the β-oxidation [54]. Malonyl-coA will be converted to acyl-coA by the enzyme FAS (transcribed by increased activity of SREBP1c). This fatty acid now has three different targets in the cell. Part of the acyl-coA produces triglyceride molecules that accumulate in the hepatocyte, leading to non-alcoholic fatty liver disease. Another amount binds to apolipoprotein (ApoB) to produce VLDL, or simply diffuses in the form of free fatty acids into the

bloodstream, triggering hypercholesterolaemia and dyslipidaemia [1,53]. The excessive influx of lipids can now reach the white adipose tissue, generating white adipose tissue (WAT) hypertrophy of it; the skeletal muscle, where it triggers insulin resistance [55]; or the pancreas, inhibiting the production and secretion of insulin. Finally, high levels of acyl-coA can be converted to diacylglycerol (DAG) by diacylglycerol acyltransferase [56]. DAG activates the protein kinase C epsilon (PKCε), which, in turn, activates the protein c-jun-*N* terminal kinase-1 (JNK1) [57]. This protein leads to hepatic insulin resistance through the phosphorylation of IRS-1 on Serine307 residue (IRS-1Ser307). This mechanism of hepatic insulin resistance perpetuates the sign of hepatic gluconeogenesis, leading to a marked increase in blood glucose and contributing to weight gain [34,58]. In 2000, Ueno and collaborators (2000) observed that insulin signaling was reduced by nearly 72% in the hepatic tissue of rodents exposed to a fructose-rich diet for 28 days [59]. In addition to inducing hepatic insulin resistance, activation of JNK-1 activates transcription factor 1 (AP-1). AP-1 transcribes inflammatory genes and activates the synthesis of inflammatory cytokines by the hepatocyte. Once released into the extracellular environment, these cytokines will bind to cytokine receptors in Kupffer cells. These cells perpetuate the inflammatory signal and can further overwhelm the hepatocyte through the release of reactive oxygen species and cytokines [60]. Both alcohol consumption and fructose consumption activate the formation of reactive oxygen species and increase the expression of inflammatory proteins in the hepatocyte, contributing to tissue damage and inflammation through this tricky process [36,61]. In addition, fructose can exist in two different stereoisomeric forms: one linear (ketone form) and the other in the form of a furanosidic ring (fructofuranose). The ratio of both forms depends on the pH and temperature of the medium. In the bloodstream, most of the fructose is in the linear form, with the ketone group exposed and susceptible to fructosylation reactions. In fact, fructose fructosylation releases large amounts of superoxide anion, leading to the disproportionate formation of reactive oxygen species [62]. The expressive increases of reactive oxygen species (ROS) leads the system to increase the antioxidant response by abruptly raising the expression of reducing proteins [63]. This response may be compromised in children and adults with micronutrient deficiency, leading to cellular and tissue damage [64]. Even under ideal micronutritional conditions, long-term administration of fructose can result in the failure of the antioxidant system [65,66]. In addition, this imbalance in the redox state and increased cell damage also lead to increases in JNK phosphorylation and activation of the AP-1 transcription factor. This pro-inflammatory additive perpetuates the insulin resistance and hepatic lipogenesis [67]. Finally, the excessive consumption of fructose produces a link between white adipose tissue and the liver [68]. Hypertrophy of white adipose tissue triggers an increased release of inflammatory cytokines by the adipocyte. Among these cytokines is the tumor necrosis factor family. In a study published by our group in 2016, we showed that levels of TNF-α in animals fed a high fructose diet were practically doubled [69]. These cytokines bind to specific cellular receptors and activate the cascade to perpetuate the inflammatory signal and insulin resistance. In the liver, these cytokines may increase the expression of another family of inflammatory receptors, the toll-like receptors (TLRs) [70], leading to overlapping systems repeating an already deregulated process of inflammatory feedback. In the skeletal muscle, the presence of these cytokines can trigger insulin resistance [69]. In the central nervous system, the presence of inflammatory cytokines prevents the efficient signaling of leptin and insulin by inhibiting the effect of these peptides on food consumption, energy expenditure, and central control of hepatic gluconeogenesis through reduced FoxO1 phosphorylation [44].

All cascades exposed so far perpetuate the harmful signal of fructose metabolism in the hepatocyte by activating a vicious cycle that can only be stopped by replacing this nutrient with another in the diet [71–73], as shown in Figure 1.

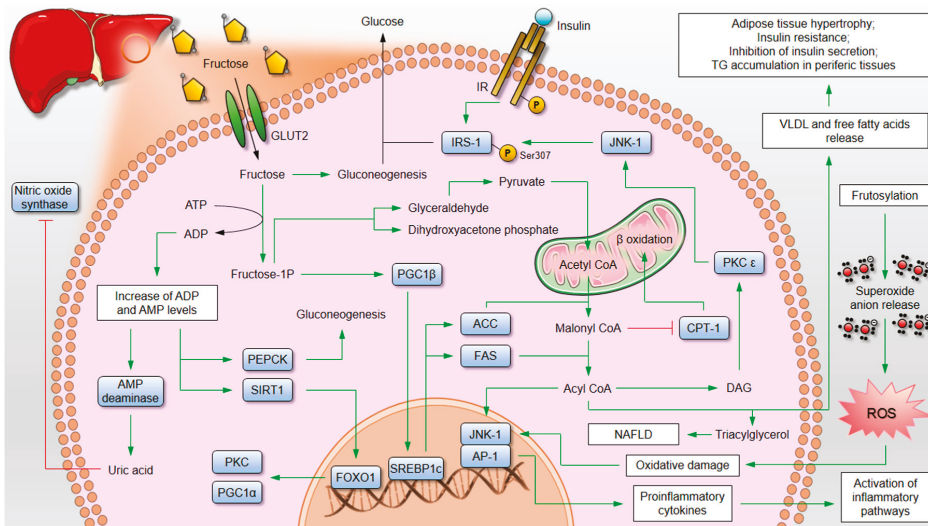

Figure 1. Role of fructose on metabolic diseases. Fructose reduces the phosphate biodisponibility, leading to acid uric production and nitric oxide synthase inhibition contributing to hypertension. Reduced phosphate biodisponibility also activates SIRT-Dependent deacetylase of FoxO1 contributing to gluconeogenesis and hyperglycemia. Fructose-1P upregulates PGC-1β expression by promoting lipogenesis through SREBP1c activation. The same nutrient provides carbon chains for the synthesis of triglycerides, diacilglycerides, and VLDL cholesterol contributing to hypertriglyceridemia, hepatic insulin resistance, and dyslipidemia. Sub products of fructose target other tissues, leading to systemic insulin resistance and inflammation. Finally, ROS generated by fructosylation increases oxidative damage and stress response in the inner of cell, leading to DNA damage and proinflammatory cytokines production. ACC: acetyil-coA carboxylase; ACC: Acetyl-CoA Carboxylase; ADP: Adenosine Diphosphate; AP-1: Activator Protein-1; ATP: Adenosine Triphosphate; CPT-1: Carnitine Palmitoyl Transferase 1; DAG: Diacylglycerol; FAS: Fatty Acyl-CoA Synthase; FoxO1: Forkhead box protein 01; Fructose-1P: Fructose 1-Phosphate; GLUT2: Glucose Transporter 2; IR: Insulin Receptor; IRS-1: Insulin Receptor Substrate 1; JNK-1: C-Jun-*N* terminal kinase-1; NAFLD: Non-Alcoholic Fat Liver Disease; PEPCK: Phosphoenolpyruvate Carboxykinase; PGC-1α: Peroxisome Proliferator-Activated Receptor-Gama Coactivator 1 Alpha; PGC-1β: Peroxisome Proliferator-Activated Receptor-Gama Coactivator 1 Beta; PKC: Protein Kinase C; ROS: Reactive Oxygen Species; SIRT-1: Sirtuin-1; SREBP1c: Sterol Regulatory Element-Binding Protein 1c; TG: Triglycerides; VLDL: Very Low Density Lipoprotein.

6. How to Deal with the Enemy

Several studies have demonstrated evidence that fructose is a nutrient with great obesogenic potential, associated with several metabolic complications and the promotion of de novo lipogenesis [1,74].

Together with obesogenic stimuli from nutritional factors, epidemiological studies also show that the genesis of obesity in contemporary society is also linked to the progressive decrease in the time available for the practice of physical activities by the global population [75]. Thus, it is strongly proposed that exercise is an important tool for combating weight gain and its associated complications, acting in the prevention and treatment of the deleterious changes promoted by high consumption of fructose. However, different models of exercise have been proposed for an improvement in metabolic health, such as aerobic exercise, strength training, and the combination of both. Therefore, we will discuss the different models separately.

6.1. Fructose Consumption and Its Complications: The Role of Aerobic Exercise

The knowledge that aerobic exercise is capable of promoting improvement in metabolic health is not recent. Studies performed at the beginning of the 20th century already provided information that physical exercise could potentiate the action of insulin, and thus increases the uptake and utilization of glucose [76]. Recently, studies using immunofluorescence staining technique demonstrated that the glucose transporters 4 (GLUT4) are stored in vesicles in the intracellular environment during rest. However, shortly after an exercise session, the GLUT4 are homogeneously redistributed by the plasma membrane, as well as when there is insulin stimulation [77]. One of the main mechanisms proposed for this phenomenon involves the activation of the protein sensitive to AMP intracellular levels, the AMP-activated protein kinase (AMPK), which is considered essential for the control of energy balance [78]. When activated, AMPK promotes the phosphorylation and activation of Akt substrate that weighs 160 kDa, the AS160. This protein, will promote the release of GLUT4, allowing the transporter to go to the cell membrane via independent mechanisms of insulin action [79,80].

Furthermore, Matos and colleagues [81] demonstrated that, when stimulated by insulin, obese animals that were submitted to an aerobic exercise session showed an insulin signaling pathway activation similar to the control group, while the sedentary obese animals showed a consistent reduction in this activation. It is suggested that this effect may be caused by the fact that aerobic exercise reduces the levels and activity of pro-inflammatory proteins [82,83], and protein-tyrosine phosphatase 1B (PTP-1B), thereby reducing the insulin resistance state [82]. Therefore, aerobic exercise increases both insulin action in skeletal muscle and glucose uptake by mechanisms that are independent of the action of this hormone. Thus, we can infer that aerobic exercise provides an agonist action of insulin in the skeletal muscle. However, the improvement of metabolic process promoted by this type of exercise is not only limited to skeletal muscle [81,82], but it also extends to other key tissues such as the liver [84,85], hypothalamus [86,87], and adipose tissue [85].

In the hepatic tissue, our group demonstrated that with only one exercise session, it is possible to reduce the levels of PTP-1B [84], a protein which is able to down-regulate insulin signal transduction [88]. The same study also found decreased levels of proteins involved in gluconeogenesis, such as PEPCK and glucose-6-phosphatase (G6Pase). Aerobic exercise also proved to be able to reduce the phosphorylation of proteins kinase RNA-like endoplasmic reticulum kinase (PERK) and eukaryotic initiation factor 2-α (eIF2α) [85], which are regarded as the greatest stress markers of endoplasmic reticulum [89]. Thus, the phosphorylation of insulin receptor tyrosine and their substrates and the activation of protein kinase B (Akt) increased, while also decreasing the inflammation in this tissue.

In the central nervous system, specifically in the hypothalamus, aerobic exercise seems to influence the control of hunger and satiety. Ropelle and colleagues [86] observed that the energy intake of animals treated with a hyperlipid diet was higher than that of animals treated with commercial feed. The authors also showed that when these same animals were submitted to an aerobic exercise session, including running and swimming, the energy intake of the obese group was equal to the control group within 12 h following the completion of the exercise. In addition, even though there was no reduction in body weight and adipose tissue, mRNA levels of pro-opiomelanocortin (POMC) were increased and the levels of neuropeptide-Y (NPY) were decreased in the hypothalamus of these animals. Rodrigues and co-authors [87] demonstrated that aerobic exercise reduced the phosphorylation and translocation of FoxO1 into the nucleus, thus inhibiting the transcription of orexigenic neuropeptides. Moreover, the protein content and activity of a mammalian homolog of Drosophila tribbles 3 (TRB3)—which may be associated with AKT and down-regulation of insulin signaling in the hypothalamus—was decreased.

Adipose tissue is also the target of molecular changes promoted by aerobic exercise. Besides reducing the amount of fat in different regions [90,91], aerobic training is also able to reduce hypertrophy of adipocytes in obese animals [91], which is essential to improve the systemic inflammatory status promoted by obesity. Notably, when hypertrophied, adipose tissue is responsible for the secretion of a series of proteins with pro-inflammatory activity [6]. Consequently, pro-inflammatory pathways such as JNK and I-kappa-B-alpha (IκBα) are less activated [85].

Once the insulin activity in several tissues that are responsible for the metabolic control has been enhanced, the serum levels of pro-inflammatory proteins [85,87], and fasting glucose [81,92] are reduced. After 8 weeks of endurance training, da Luz and colleagues [85] observed a decrease in serum levels of TNF-α, demonstrating that there was a systemic decrease in the inflammation of obese animals. Likewise, aerobic exercise also reduces oxidative stress and increases antioxidant capacity.

Aerobic exercise is also proposed as a strong strategy for the prevention and treatment of NAFLD. Gauthier and colleagues [93] found that sedentary obese animals had a 72% increase in fat accumulation in the liver, with 48% more lipid vacuoles than the animals treated with a standard diet. However, animals that performed aerobic exercise during the obesity induction period had a reduction in the development of NAFLD. Similar results were found by Shen and colleagues [91], in which the amount of fat observed in the liver of obese and exercised animals was similar to that observed in the control animals. These results were still accompanied by a reduction in the gene expression of stearoyl-CoA desaturase-1 (SCD-1), described as a key regulator of lipid metabolism, so that this deletion provides an improvement in the oxidation of fatty acids machinery in the liver [94]. Charbonneau and colleagues [90] also observed that training the obese animals resulted in equating the liver TG levels to those in the control group.

As discussed previously, the lipogenic properties of fructose are associated with large increases in triglyceride levels [95–97]. In this context, the practice of exercise, especially aerobic exercise, has been shown to promote important and consistent effects related to the pathogenesis of dyslipidaemia [92,98]. In an important meta-analysis involving 51 studies, Leon and Sanchez [99] found that after 12 weeks or more of aerobic exercise intervention, the subjects had a mean reduction of 3.7% in triglyceride levels, while high-density lipoprotein (HDL) levels were elevated by 4.6% and low-density lipoprotein (LDL) was reduced by 5%, on average.

6.1.1. Animal Evidence

Finally, there is evidence that aerobic exercise is an important tool to combat various metabolic complications induced by high fructose consumption [69,100]. In a study conducted by Stanišić and colleagues [101], Wistar rats received a 10% fructose solution for 8 weeks, and thereafter a significant increase in insulin levels and severe insulin resistance were observed. However, animals that underwent a running exercise on a treadmill during the experiment had such attenuated complications. Farah and colleagues [102], using the same intervention, observed that animals who received a fructose solution presented glucose intolerance and increased adiposity; however, the practice of moderate aerobic exercise was effective to attenuate these complications.

Our research group [69] showed that aerobic exercise, in addition to reducing the activity of inflammatory proteins in the skeletal muscle of animals fed a high fructose diet, also increased levels of interleukin 10, described as a protein with a potent anti-inflammatory potential [103]. Exercised rodents treated with fructose also had a decrease in oxidative stress markers [102] and NAFLD [100], and a lower TG accumulation in liver tissue [69]. Moreover, Farah and colleagues [102] showed that aerobic exercise was able to prevent hemodynamic and autonomic cardiac dysfunction promoted by high fructose diet. On the other hand, Karaca and colleagues [104] demonstrated that positive adaptations promoted by physical exercise such as increased expression of aquaporin 7 (AQP7), an important protein related to metabolic health of cardiac tissue during exercise, were inhibited by fructose-rich diets.

6.1.2. Human Evidence

Similar to animal models, studies in humans have shown that aerobic exercise is an efficient strategy for inhibiting the harmful effects of high fructose consumption. A study involving healthy humans revealed that even after the daily addition of 75 g of fructose in their diet, physical activity was able to reduce insulin levels and maintain unchanged the triglyceride levels of active volunteers, while, on the other hand, the inactive people showed an increase in this parameter [105]. In addition,

Egli and colleagues [106] observed that aerobic exercise (moderate intensity) completely eliminated the deleterious effects promoted by 4 days of diet consumption composed by 30% of fructose.

6.2. Fructose Consumption and Its Complicatons: The Role of Strength Exercise

Although we are reaching a century of publications on metabolic syndrome related to aerobic exercise, the same does not apply to strength exercise. In 1984, Miller and colleagues [107], in a longitudinal study of healthy subjects, observed the effects of completion of 10 weeks of isotonic exercise with weights. After the training protocol, participants showed no differences in fasting glucose, but the plasma insulin levels were significantly reduced, from 10.85 ± 1.55 μU/mL to 6.79 ± 1.19 μU/mL. During the glucose tolerance test, the area under the insulinemic curve of subjects submitted to resistance training was also lower than that of those who remained sedentary during the same period, thus bringing the first evidence that strength exercise also has the capacity to improve insulin sensitivity. Finally, the authors also observed an increase in the amount of muscle mass in the trained group, and that these gains had a strong negative correlation with insulin levels during glucose tolerance test. A decade later, Treuth and colleagues [108] showed evidence on the relationship of strength training with adiposity control and, consequently, obesity. After 16 weeks of exercise performed three times per week, the authors observed that although the body weight of participants was not changed, the amount of intra-abdominal adipose tissue was significantly reduced [109].

Recent studies have provided us with a better understanding of how strength exercise is able to improve glycemic control. A performed study which used obese animals and submitted them to strength exercise on staircase [110], besides confirming reductions in serum insulin levels, showed that after 8 weeks of training, some proteins from the insulin pathway had increased their action, such as phosphatidylinositol 3-kinase (PI3-K) and Akt. Furthermore, the GLUT4 gene expression was also increased in these animals. Another study published by the same group of researchers [111] showed that serum levels of TNF-α and interleukin-6 were reduced with training, accompanied by an increase of adiponectin [112]. However, obese subjects undergoing strength training showed improvement in insulin sensitivity even without changes in serum levels of proinflammatory cytokines [113].

Several studies have begun to suggest strength exercise as an important strategy for the prevention and treatment of obesity [114–116] and in one of these studies Schmitz and colleagues [115] found positive results in body composition of women who performed strength exercise. In the fifteenth week of intervention, there was a reduction of body fat and an increase in muscle mass. In another state, after one year of intervention, a reduction in the amount of intra-abdominal fat was also observed [116].

After a strength exercise session, positive changes on the lipid profile were observed by Lira and colleagues [117]. However, interestingly, different responses were observed according to the intensity of the exercise. After 72 h of the end of the session, the plasma TG levels were reduced only in subjects who trained in the intensities of 50% and 75% of 1 RM, while those who trained at 90% and 100% showed no differences. Also, after an exercise session, the sensitivity to insulin in the hepatic tissue was improved by $8\% \pm 1\%$ reductions in glucose production rate and $12\% \pm 5\%$ glycogenolysis [118].

6.2.1. Animal Evidence

Strength exercise is not as common of an intervention in animal models as aerobic exercise. In a review, Seo and colleagues [119] identified several reasons that hindered the use of this intervention in animals. Strength exercise requires animal voluntary performance, and variables such as intensity and time between sets are difficult to control, thus, the results obtained by these studies are often not very expressive and may be misinterpreted. Because of this, only one study involving strength exercise and fructose consumption was found, and it was carried out by our research group [69]. In summary, the strength exercise promoted consistent improvements in the metabolism of rodents fed a high fructose diet. Similar to aerobic exercise, resistance exercise provided lower levels of glucose and insulin during glucose tolerance test. Moreover, strength exercise decreased interleukin-6 (IL-6) and TNF-α levels. Regarding the liver tissue, strength exercise decreased fat accumulation and

led to a greater reduction in the levels of nuclear factor-kappa B (NF-κB) and IκB-α, demonstrating that strength exercise can be promising for the reduction of inflammation promoted by a diet rich in fructose.

6.2.2. Human Evidence

As previously discussed, there are fewer publications about strength exercise than aerobic exercise. The only study that investigated the acute effects of strength exercise with high fructose diet in humans was carried out by Wilburn and colleagues [120]. On the first day of intervention, participants performed 14 different strength exercises (4 sets of 10 repetitions each with 90 s rest between sets) during 95 min. After 15 h of the training session, participants were fed a meal containing 0.75 g/kg body weight (BW) of fructose, and several blood samples were taken for the next 360 min. Although insulin and lactate levels did not differ between the trained and sedentary group after the fructose-rich meal, the postprandial TG concentrations were significantly lower in the trained group. Based on this evidence, the authors concluded that strength exercise performed prior to fructose-rich meals might attenuate elevations in serum TG levels; however, further studies are needed to better understand this issue.

6.3. Fructose Consumption and Its Complicatons: The Role of Combined Exercise

In the last few decades, the effects of combined physical exercise have been gaining prominence in the scientific community. This exercise protocol consists of endurance and strength exercises performed in the same training session or on alternate days. Initially, the practice of combined exercise did not seem to be an interesting strategy for metabolic health, since in the 1980s, a classic study revealed that when subjects performed strength exercises and subsequently practiced cycling and running, the strength gains, muscle mass, and consequent increase in body weight were compromised [121]. However, the gains in aerobic performance were not different between individuals who performed aerobic training and those who underwent the combined training. A few years later, the results found by Kraemer and colleagues [122] corroborated the above article, where the authors found that subjects who underwent the combined exercise showed a smaller area in the muscle fibers than those who performed only strength exercise. Thus, it was also shown that combined exercise not only compromised the strength gain promoted by strength exercise, but also hypertrophied different muscle fibers.

However, subsequent studies began to demonstrate evidence that challenged the negative influence of combined exercise on strength gain and cross-sectional area on muscle fibers. In their study, McCarthy and colleagues [123] showed that the combined exercise, performed three times a week for 10 weeks, promoted the same increase in flexor and knee extensor muscle area as strength exercise alone, with no difference in neural activation. In a study by De Souza and colleagues [124], although they did not find hypertrophy after the combined training protocol, they did not find differences in the expression of genes related to the control of protein synthesis, suggesting that the control of other training variables, such as session volume, may be related to these different responses found in the different protocols. Finally, it has also been demonstrated in humans that the activation of proteins involved in protein synthesis, such as the mechanistic target of rapamycin (mTOR) and ribosomal protein S6 kinase beta-1 (S6K1), are not compromised. In fact, AMPK activation [125], a protein that has an increased activity caused by the increase in AMP levels provided by aerobic exercise [83,126], was shown to be able to negatively influence the stimulation of protein synthesis by inhibiting the mTOR pathway in rodents [127]. From this, many other studies have been conducted to better understand the changes promoted by this new and promising exercise protocol, in many different contexts, because it is believed that this type of exercise can activate different pathways and promote several improvements. For these reasons, currently, the practice of combined exercise is widely recommended by the American College of Sports and Medicine [128].

Monteiro and colleagues [129] observed positive effects on body composition in obese adolescents after 20 weeks of combined exercise. At the end of the experiment, the participants presented a reduction in body weight and body mass index (BMI). Interestingly, the magnitude in reducing body fat was very close between the two groups who underwent the combined exercise and aerobic exercise only, and this reduction was 3.5% and 3.9% respectively. Moreover, consistent results were also found in the lipid profile, with reductions in circulating levels of TG and VLDL and increases in HDL levels. On the other hand, the waist circumference of these participants was not different.

Medeiros and colleagues [130] also found positive results of combined exercise on body composition in obese subjects, reflecting an improvement in insulin resistance and oxidative stress markers. Interestingly, the authors used two groups with similar exercise protocols, but one group exercised three times a week, while other exercised 5 times a week. Both groups showed a reduction in body weight and BMI. The group that exercised five times per week reduced the amount of fat mass and increased the amount of muscle mass. The group that performed the training protocol three times per week presented reductions in fasting blood glucose levels and the homeostasis model assessment-estimated insulin resistance (HOMA-IR) index. Reductions in protein carbonylation levels were observed in the group that performed three weekly sessions, indicating an improvement in the antioxidant machinery, since this is a process triggered by ROS [131]. The activity of glutathione peroxidase was decreased and that of lipid peroxidation was increased in both groups. It is known that exercise can moderately increase ROS production, and thus provides positive adaptations for the entire antioxidant system [132]. Thus, these results suggest that combined exercise may be an important strategy for combating insulin resistance and oxidative stress associated with obesity, but more studies are needed to fully understand controls of exercise variables, such as exercise intensity and frequency.

Finally, the combined exercise was also able to present improvements in several deleterious contexts promoted by the high consumption of fructose [69]. Our research group revealed that combined exercise, when performed on alternate days (aerobic and strength on separate days), despite not promoting reduction in body weight, was able to provide an increase in glucose tolerance and insulin sensitivity compared to the aerobic protocol. The circulating levels of HDL were increased, and the reduction of hepatic TG accumulation was strongly visible in histological sections, when compared to the sedentary animals that received high doses of fructose. Finally, exercise also provided a lower activation of Nuclear factor-kappa B (NF-κB), thus presenting a decrease in systemic inflammation. There are no studies on humans that have investigated the role of combined exercise to attenuate the deleterious effects of high fructose diet, thus more studies are needed to fill this literature gap.

7. Conclusions

The consumption of fructose has been increasing along with its harmful effects on the organism. Fructose may trigger changes in the circulating and hepatic lipid profile, favoring the installation of chronic and subclinical inflammation. Regular practice of aerobic physical exercise, strength training, or a combination of both, in turn, has the ability to reverse these parameters, mainly by improving the circulating and tissue lipid profile and reducing inflammation (Figure 2). Therefore, regular practice of physical exercise is an essential tool for attenuating the obesogenic disorders caused by the consumption of fructose.

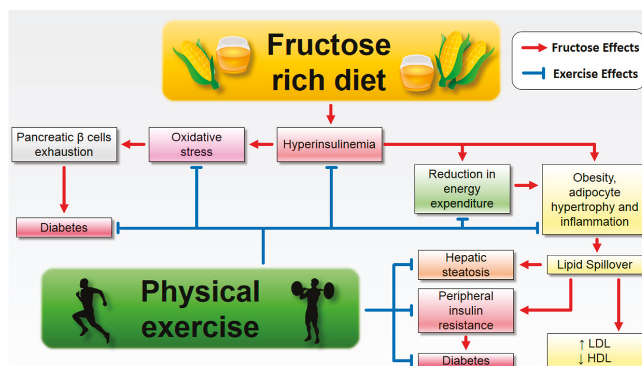

Figure 2. Exercise prevents and treats the deleterious effects of high consumption of fructose. In addition to promoting an increase in energy expenditure, physical exercise consistently attenuates inflammation and oxidative stress related to excessive consumption of fructose, as reflected in positive changes both in lipid profile and fat metabolism. In this way, insulin resistance and hyperinsulinaemia are diminished, collaborating with the prevention and treatment of diseases such as hepatic steatosis and type 2 diabetes. HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein.

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Abbreviations

ACC	Acetyl-CoA Carboxylase
ADP	Adenosine Diphosphate
Akt	Protein kinase B
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
AP-1	Activator Protein-1
AQP7	Aquaporin 7
ATP	Adenosine Triphosphate
BMI	Body mass index
CB1	Cannabinoid 1
CPT-1	Carnitine Palmitoyl Transferase 1
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
eIF2α	Eukaryotic initiation factor 2-α
FAS	Fatty Acyl-CoA Synthase
FoxO1	Forkhead box protein 01
Fructose 1-P	Fructose 1-Phosphate
G6Pase	Glucose-6-phosphatase
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GLUT5	Glucose Transporter 5
HDL	High-Density Lipoprotein
HFCS-42	High-Fructose Corn Syrup with 42% of Fructose

HFCS-55	High-Fructose Corn Syrup with 55% of Fructose
HFCS	High-Fructose Corn Syrup
IκBα	I-kappa-B-alpha
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate 1
JNK 1	C-Jun-N terminal kinase-1
LDL	Low-Density Lipoprotein
mTOR	Mechanistic target of rapamycin
NAD^+/NADH	Nicotinamide Adenine Dinucleotide
NAFLD	Non-Alcoholic Fat Liver Disease
NF-κB	Nuclear factor-kappa B
NPY	Neuropeptide-Y
PEPCK	Phosphoenolpyruvate Carboxykinase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1α	Peroxisome Proliferator-Activated Receptor-Gama Coactivator 1 Alpha
PGC-1β	Peroxisome Proliferator-Activated Receptor-Gama Coactivator 1 Beta
PKC	Protein Kinase C
POMC	Proopiomelanocortin
PTP-1B	Protein-tyrosine phosphatase 1B
ROS	Reactive Oxygen Species
S6K1	Ribosomal protein S6 kinase beta-1
SCD-1	Stearoyl-CoA desaturase-1
SIRT-1	Sirtuin-1
SREBP1c	Sterol Regulatory Element-Binding Protein 1c
TG	Triglycerides
TNF-α	Tumor necrosis factor alpha
TRB3	Tribbles homolog 3
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue

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Review

Fructose, Glucocorticoids and Adipose Tissue: Implications for the Metabolic Syndrome

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Abstract: The modern Western society lifestyle is characterized by a hyperenergetic, high sugar containing food intake. Sugar intake increased dramatically during the last few decades, due to the excessive consumption of high-sugar drinks and high-fructose corn syrup. Current evidence suggests that high fructose intake when combined with overeating and adiposity promotes adverse metabolic health effects including dyslipidemia, insulin resistance, type II diabetes, and inflammation. Similarly, elevated glucocorticoid levels, especially the enhanced generation of active glucocorticoids in the adipose tissue due to increased 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) activity, have been associated with metabolic diseases. Moreover, recent evidence suggests that fructose stimulates the 11β-HSD1-mediated glucocorticoid activation by enhancing the availability of its cofactor NADPH. In adipocytes, fructose was found to stimulate 11β-HSD1 expression and activity, thereby promoting the adipogenic effects of glucocorticoids. This article aims to highlight the interconnections between overwhelmed fructose metabolism, intracellular glucocorticoid activation in adipose tissue, and their metabolic effects on the progression of the metabolic syndrome.

Keywords: fructose; glucocorticoid; obesity; metabolic syndrome; adipogenesis

1. Introduction

Our ancestors obtained their food from hunting and gathering, but the transition to modern Western society lifestyle with its tremendous technological advances in food processing led to extensive changes in food intake and composition. The Western-style diet, also called the meat-sweet diet is characterized by high intakes of processed foods rich in saturated fat, trans-fatty acids, proteins from red meat, and sodium, as well as an excessive consumption of sugar [1]. In line with this transition, obesity has emerged as a major global health problem in the last few decades [2]. Epidemiologic studies pointed out that overweight and obesity are important risk factors of type II diabetes mellitus (T2DM) and cardiovascular disease (CVD) [3–6]. The involvement of adiposity—predominantly splanchnic obesity—in the development of the metabolic syndrome (MetS) has been well established [7]. Metabolic and endocrine factors, like hormones and para/autocrine mediators, have been shown to stimulate adipocyte proliferation and differentiation [8]. When the adipose tissue reaches a critical mass and/or hypoxia occurs, a cellular signaling response triggers a switch from oxidative metabolism

to anaerobic glycolysis and increases the secretion of a number of inflammation-related adipokines accompanied with cell damage [9]. On the other hand, this inflammatory response is also a key factor for modulating insulin sensitivity in adipose tissue and the development of obesity-associated diseases [10]. The chronic low-grade inflammation is a characteristic of obesity, whereby adipose tissue releases many inflammatory mediators, including TNF-α, IL-1β, and IL-6, and plasma concentrations of these secreted pro-inflammatory cytokines were found to be elevated in obese individuals [11].

The rate of dietary fructose consumption, mostly in combination with glucose, continued to rise worldwide over the last fifty years [12,13], and numerous human and animal studies demonstrated a link to the rising prevalence of obesity, T2DM, and MetS [14–17]. Fructose, which is found in fruits, became a major component of the modern diet by robust intake of sucrose (table sugar, consisting of one molecule of glucose and one molecule of fructose and subjected to cleavage in the intestinal tract) and synthetic high fructose corn syrup (HFCS, consisting of a mixture of glucose and fructose with a ratio close to one) [18] that is currently added to beverages and foods. Compared with glucose, fructose has a lower glycemic index, does not generate an insulin response, but has a slightly higher sweetening power. Furthermore, fructose is a potent lipogenic and adipogenic nutrient. For example, fructose rich diet intake increased the adipogenic potential on adipocyte precursor cells (APCs) and hence accelerated adipocyte hypertrophy [19].

Besides dietary fructose, an increased intracellular glucocorticoid production, especially in adipose tissue, has also been suggested to contribute to the pathogenesis of the MetS [20–22], and evidence was provided for a possible link between fructose and glucocorticoid activation [23–25]. The circulating and locally produced glucocorticoids have a crucial role in modulating adipocyte function as well as proliferation/differentiation [26,27]. The intracellular active glucocorticoids (cortisol and corticosterone are the major glucocorticoids in humans and rodents, respectively) are generated from their inert forms (cortisone and 11-dehydrocorticosterone) by the 11-oxoreductase activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [28,29], a luminally oriented enzyme of the endoplasmic reticulum (ER) membrane [30,31], which is abundantly expressed in liver and adipose tissue [32–34].

In the adipocyte, fructose metabolism results in the generation of precursors of fatty acid synthesis and induces NADPH-generating enzymes. Recent observations indicated that fructose increases the expression of its transporter GLUT5 in the adipocyte plasma membrane and of 11β-HSD1 [35], thereby further enhancing the capability to generate active glucocorticoids in adipose tissue [25].

The purpose of the present work was to highlight the multifaceted connections between fructose metabolism and the production of active glucocorticoids in the adipose tissue and its impact on the development and progression of MetS.

2. Dietary Fructose and Adiposity

Epidemiological studies have linked dietary fructose consumption, either in the form of sucrose or HFCS, with an increased rate of co-occurring diseases of the MetS, such as CVD, T2DM, and non-alcoholic fatty liver disease (NAFLD) [36–39]. A cross-sectional study among adults from the National Health and Nutrition Examination study (NHANES) 1999–2006 found an association between the consumption of dietary added sugars, as assessed by 24 h dietary recall, and blood lipid measures, with significant increases in mean triglyceride (TG) levels and decreases in high-density lipoprotein (HDL)-cholesterol levels [36]. This study including more than 6000 adults, did not distinguish between fructose and glucose consumption but investigated associations with total dietary sugar consumption. Another study, using econometric models of repeated cross-sectional data on diabetes and nutritional components of food, reported on an association of a high sugar intake with T2DM, an effect that was modified but not confounded by overweight or obesity and that was not dependent on a sedentary lifestyle [37]. Furthermore, an analysis of dietary history and paired serum and liver tissue from patients with NAFLD and gender, age, and body mass index matched controls revealed a 2–3 fold higher fructose consumption (in the form of HFCS) in NAFLD patients with increased hepatic fructokinase (ketohexokinase, KHK) and fatty acid synthase expression, indicating elevated

lipogenesis [38]. Dietary information was prospectively collected. Especially the consumption of sugar sweetened beverages have been regarded as critical for development of obesity, hypertension, and T2DM [14,40–43]. Another recent observational clinical study revealed increased visceral adipose tissue (VAT) using computer tomography in 1003 participants in response to sugar-sweetened beverages [44]. In this large, prospective cohort study the participants were categorized according to sugar-sweetened beverage intake frequency (non-consumers; <1 serving/week; <1 serving/day and daily consumers) and examined the adverse changes in quality and quantity of VAT after a period of six years. The study concluded that fructose, as the main component, may trigger insulin resistance and increased fat accumulation in VAT found in consumers of excessive amounts of sugar-sweetened beverages. However, it remains entirely uncertain whether the fructose or the glucose component of the sweetener or both are responsible for the metabolic effects associated with sugar-sweetened beverage consumption. Also, the pathophysiological mechanisms involved in the process contributing to the increased risk of T2DM and CVD remain to be elucidated.

An earlier clinical study evaluated the relative effects of the consumption of glucose- and fructose-sweetened beverages in overweight and obese individuals, where these beverages covered 25% of the total energy requirements for 10 weeks [45]. Although both sweetener beverages exhibited similar weight gain, the results showed that consumption of fructose-sweetened but not glucose-sweetened beverages increased de novo lipogenesis, specifically promoting lipid deposition in VAT, stimulating dyslipidemia, altering lipoprotein remodeling and decreasing insulin sensitivity in overweight/obese adults [45]. It is still uncertain whether the observed effects of this study are comparable with the results when pure fructose-sweetened beverages are consumed, or when isocaloric fructose and/or glucose are combined with different fat diets. A critical issue remains that fructose consumption and obesity are linked and that so far no clear association between fructose intake and cardiometabolic disease has been demonstrated conclusively in the absence of overeating and weight gain [40,46–48]. According to another concept, fructose could contribute to obesity by stimulating sterol receptor element binding protein 1c (SREBP-1c) independently of insulin, which activates genes involved in de novo lipogenesis [49], generating fatty acids for TG production in the liver. An increase in fasting plasma TG has been observed upon excessive dietary fructose ingestion in healthy individuals as well as patients with T2DM [50,51]. Furthermore, increased hepatic lipid levels are associated with increased very low density lipoprotein (VLDL) synthesis and secretion. An elevation of systemic free fatty acid and VLDL results in increased lipid uptake in peripheral organs, such as adipose tissue and skeletal muscle, contributing to the systemic insulin resistance [15]. Additionally, fructose was proposed to promote leptin resistance, worsening obesity and insulin resistance [52]. Insulin resistance may be a secondary cause of obesity upon consumption of a hyperenergetic and high fructose-containing diet [53,54], again pointing to the importance of dissecting the direct impact of fructose and the consequences of overeating and obesity. Thus, further studies are needed to address the effects of fructose and sucrose intake under isocaloric dietary regimens and in defined subgroups, including patients with obesity, T2DM, and CVD.

An obesogenic effect of large doses of fructose was also observed in animal studies. Rats fed a diet consisting of 60% of energy derived from fructose [55], and rhesus monkeys on a daily fructose intake of 30% of ingested calories [56] had increased adipose tissue weight and several other features of MetS [57]. The fructose fed monkeys, besides adiposity, also displayed dyslipidemia, insulin resistance, and enhanced inflammatory mediators. While existing evidence convincingly shows that markers of MetS including TG, dyslipidemia, insulin resistance, and inflammatory mediators are enhanced upon high fructose containing diets [46–48], no consistent effect of fructose on markers of MetS could be found in studies using a defined weight-maintenance diet [47], suggesting that high fructose ingestion combined with overeating and adiposity may be responsible for the adverse health effects.

3. Fructose Metabolism in Adipose Tissue

Upon intestinal absorption, fructose is primarily metabolized by KHK to fructose-1-phosphate in the liver, which is subsequently converted to triose phosphates and in this form can supply glycolysis, lipid synthesis, gluconeogenesis, and/or glycogenesis pathways [58]. Fructose metabolism to triose phosphates differs from that of glucose: it occurs independently of insulin and without the negative feedback regulation of phosphofructokinase in the glycolytic pathway. The excessive consumption of fructose challenges the capacity of the liver, and to a lesser extent fructose remains in the systemic circulation, resulting in its utilization in peripheral tissues. Although adipocytes express GLUT5 [59] and are able to take up fructose [60], the functional role of this fructose transporter in the adipose tissue is not fully understood. In this regard, a novel significance of fructose and GLUT5 was pointed out in regulating adipocyte differentiation [61]. Furthermore, it is known that the pathway of fructose utilization in adipose tissue is largely different from that of the hepatic metabolism (Figure 1). In contrast to hepatocytes, adipocytes lack KHK and are equipped with hexokinase, which catalyzes the phosphorylation of fructose to fructose-6-phosphate (F6P) [62]. This obligatory intermediate, F6P, can be converted to glucose-6-phosphate (G6P) by G6P isomerase in the cytoplasm and the ER [25], or can be further metabolized by intermediary metabolic pathways. Investigating these roads and predicting the metabolic responses of adipocytes to fructose is challenging.

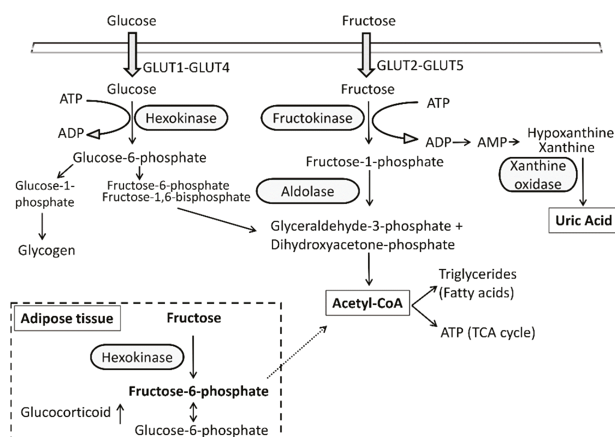

Figure 1. Intracellular metabolism of fructose and glucose. The intracellular metabolism of fructose differs from that of glucose primarily due to its different transporters and initial enzymatic steps. The main fructose metabolizing enzyme is fructokinase (ketohexokinase, KHK), which uses ATP to phosphorylate fructose to fructose-1-phosphate. Since this reaction is poorly regulated, the administration of excessive fructose results in rapid depletion of intracellular ATP levels, activation of AMP deaminase, and generation of uric acid. In adipocytes due to the lack of fructokinase, fructose is metabolized by hexokinase to fructose-6-phosphate, which can be converted to glucose-6-phosphate that can promote the intracellular production of glucocorticoids via stimulation of 11β-HSD1 activity.

An important experimental model represents the use of ^{13}C labeled fructose for in vitro and in vivo investigations. A recent study was performed using a stable isotope based dynamic profiling (SIDMAP) method with labeled [U-$^{13}C_6$]-D-fructose in differentiating and differentiated adipocytes (Simpson-Golabi-Behmel Syndrome (SGBS) adipose cells) exposed with an escalating range of fructose equivalent to predict the metabolic responses in detail [63]. Varma and colleagues found that increasing concentrations of fructose triggered the pyruvate conversion to acetyl-CoA via the pyruvate dehydrogenase reaction to form glutamate. The pyruvate dehydrogenase flux derived increased entry into the TCA cycle also resulted in an expanded acetyl-CoA/citrate cycling into fatty acid synthesis and

free palmitate release [63]. These results explored the lipogenic potential of fructose in adipocytes and how fructose acts as an anabolic substrate for molecular synthesis and energy storage and much less so for oxidation. On the other hand, when the intermediary metabolism of glucose was investigated with the same method using [1,2-$^{13}C_2$]-D-glucose under the influence of increasing concentrations of fructose, their data showed that fructose dose-dependently increased the oxidation of glucose, triggered the conversion of glucose to lactate, but decreased the formation of glutamate or glycogen from glucose and reduced the potential route for fatty acid synthesis and ribose synthesis [64]. The main novelty of that study was to discover the role of the recently described serine synthesis, one-carbon cycle, and glycine cleavage (SOGC) pathway in the fate of glucose carbons in the presence of added fructose in adipocytes. In this pathway, the glucose derived glycolytic metabolite 3-phosphoglycerate is used for the synthesis of serine, from which a fraction subsequently is converted to glycine in a reaction that is coupled with the one carbon metabolism pathway, yielding ATP [65]. The intermediates of the one carbon pathway generate NADPH, a key cofactor needed for fatty acid synthesis. Thus, the presence of fructose in adipocytes drives this alternate pathway, resulting in increased energy and CO_2 production, which can be utilized in fructose-induced lipogenesis and fat storage in adipocytes [64].

A recent in vivo study supported the above described effects of fructose. Independent of whether fructose was provided either as a monosaccharide or in the form of sucrose combined with glucose, fructose increased the whole-body exogenous carbohydrate oxidation rate during prolonged exercise in volunteer healthy and trained cyclists [66]. A higher exogenous glucose oxidation rate was found to correlate with increased performance during prolonged high-intensity exercise [67] and co-ingestion of fructose further enhanced exogenous carbohydrate oxidation rates [66] and decreased gastrointestinal distress [68,69]. In type 1 diabetic individuals, the co-ingestion of glucose and fructose was also found to be beneficial during exercise compared to glucose alone. However, carbohydrate oxidation was lower but fat oxidation was higher upon co-ingestion of fructose and glucose compared to glucose alone in diabetic patients during exercise [70]. Additionally, the diabetic patients showed a glycogen-sparing effect in the working muscle, although their lactate production was elevated, as was also described previously in healthy individuals [71]. This suggests that the ingested fructose might be partially converted into lactate [72], or the fructose itself prompts conversion of glucose into lactate, as seen in vitro [64]. To conclude, these studies showed that co-ingestion of fructose with glucose may optimize fuel metabolism during exercise in healthy individuals by a more efficient energy supply due to higher carbohydrate oxidation and in diabetic patients by increased fat oxidation.

4. Effect of Fructose on Metabolic Disturbances

In the last couple of decades, the cellular and molecular mechanisms of adipocyte differentiation have been extensively studied and various hormones and growth factors affecting adipocyte differentiation in a positive or negative manner have been identified [9,73–78]. Although several clinical and in vitro studies defined the high lipogenicity of fructose and its stimulation of adipogenesis [19,79,80], further questions were raised on the exact mechanisms and regulatory factors involved behind this phenomenon.

There is compelling evidence that oxidative stress is implicated in fructose-mediated adiposity, insulin resistance, and metabolic syndrome. In a study in rats, fructose induced the mRNA and protein expression of ER stress markers, including GRP-78, PERK, IRE1α, and CHOP in the liver [81]; which on one hand might contribute to the hepatic activation of SREBP-1c and lipid accumulation in fructose-induced NAFLD [38], and on the other hand the increased ER-stress is also suggested to cause hepatic insulin resistance by increasing de novo lipogenesis [10,82]. Another central role of the ER is to control the transport and metabolism of cholesterol, an essential component of cellular membranes, which is mainly regulated by transcription factors of the SREBP family [83].

Another aspect of the fructose-mediated metabolic effects, when it is rapidly metabolized in the liver, is its conversion to fructose-1-phosphate, which can cause intracellular phosphate depletion and AMP formation (Figure 1), resulting in the activation of AMP deaminase and the formation of uric

acid [58]. Uric acid production has been identified as a sensitive measure of hepatic ATP depletion [84]. The serum uric acid can rapidly raise, up to a level of 2 mg/dL, after intravenous or oral fructose consumption [85,86]. Although this initial increase is transient, it was found that administration of fructose for several weeks increases fasting uric acid levels [87,88]. Elevated levels of uric acid have been associated with a series of pathological conditions, including insulin resistance, obesity, T2DM, and chronic kidney disease [57,89] and have been proposed as a risk factor for myocardial infarction and neurological diseases including stroke [90,91]. Lowering the uric acid level using the xanthine oxidase inhibitor febuxostat prevented the fructose-induced development of MetS [92]. One suggested mechanism includes the direct effect of uric acid on adipocytes. Using cultured adipocytes, evidence was provided for an induction of oxidative stress and inflammation by uric acid [93]. Knockdown of the xanthine oxidoreductase inhibited adipogenesis and PPARγ activity [94]. These studies implicate that uric acid might serve as an important regulator of adipogenesis; therefore, fructose-mediated uric acid formation might be associated with insulin resistance and MetS.

It is important to note that rats possess uricase, which degrades uric acid to allantoin, explaining why fructose does not increase the uric acid level effectively in this species. In experiments with rats, uricase inhibitors are needed; however, this leads to more than 10-fold increased uric acid levels in response to fructose administration [95]. In contrast, humans have no functional uricase due to an evolutionary mutation [96]. Another reason why the rat may be of limited relevance to study fructose toxicity is the fact that rats can produce ascorbate (vitamin C), which can block the adverse effects of fructose. As an antioxidant, vitamin C can attenuate uric acid-mediated vascular smooth muscle cell effects and hypertension [97,98].

In the adipose tissue, ER-stress induction also plays an important role in the pathomechanism of impaired differentiation processes [99]. During maturation, the ER environment of fibroblast-like preadipocytes must confront the demand of secreting enormous amounts of peptide and lipid mediators and storing energy in the form of TG in lipid droplets [100]. During nutrient overload and/or disturbances, cellular stress can lead to an impairment of ER function, limiting the capacity of proper protein folding and resulting in an accumulation of unfolded proteins in the ER lumen, ultimately leading to impaired adipocyte maturation. Such a mechanism for fructose-mediated ER-stress induction in adipocytes was reported recently by Marek et al. [101]. They provided important mechanistic insight into how fructose consumption not only influences ER redox status via depleting ERO-1α expression but also affects one of the key ER-stress signaling pathways by inducing XBP-1 splicing in the VAT of treated mice. Since the assembly and secretion of the beneficial anti-inflammatory and insulin-sensitizing adipokine adiponectin is regulated by ER chaperones such as ERO-1α and ERp44 [102], the fructose-mediated depletion of the biologically active high-molecular weight protein adiponectin might be explained by an altered ER homeostasis [101]. Furthermore, this study revealed macrophage infiltration and increased expression of inflammatory cytokines such as monocyte chemoattractant-1 (MCP-1) and tumor necrosis factor-α (TNF-α) in the VAT in response to fructose [101]. Importantly, all of these fructose-mediated effects were mediated by KHK. Although, the low-activity KHK (KHK-A) isoform is expressed in adipocytes [103] and alternative hexokinase-mediated fructose metabolism takes part in adipose tissue, it is more reasonable to imply that the observed fructose-mediated metabolic effects are triggered by KHK-C-dependent metabolism in the liver. These recent results suggest that the enhanced hepatic de novo lipogenesis and TG production affect adipose tissue via intermediary metabolic and inflammatory communication. The fructose-induced proinflammatory process with infiltrated macrophages in VAT and the caused adiponectin resistance are the main important contributors to insulin resistance and global metabolic changes in the situation of fructose over-consumption [101].

Regarding this hypothesis, an important role of the ER protein CHOP in modulating the polarity of adipose tissue macrophages was proposed recently [104]. A high fat diet (HFD; consisting of saturated fat, protein, and sucrose, i.e., 32% sunflower oil, 33% casein, 18% sucrose) resulting in the induction of ER-stress led to upregulation of CHOP expression in adipocytes, altering adipocyte

function and suppressing microenvironment conditions, involving downregulation of Th2 cytokines needed to inhibit M2 polarization of macrophages infiltrated in the adipose. Hence ER-stress induction results in chronic inflammation in adipose tissue and insulin resistance at the whole-body level. In mice with CHOP deficiency, adipose tissue macrophage M2 polarization was maintained and these mice were protected against HFD-mediated metabolic effects and insulin resistance [104].

Another important mediator of adipogenesis and adipocyte function includes glucocorticoids. Although both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) are expressed in adipose tissue and have roles in regulating leptin expression, silencing experiments showed that GR has more important roles in mediating adipogenesis and adipokine production in human adipocytes [74,105]. In addition, the intracellular generation of active glucocorticoids in monocyte/macrophages regulates the release of pro-inflammatory molecules [106]. It was shown that the selective inhibition of 11β-HSD1 improved multiple MetS parameters, suppressed the inflammation of adipose tissue [107], exerted anti-inflammatory effects in lipopolysaccharide (LPS)-activated macrophages via the stimulation of heme oxygenase-1 [108], and reduced pro-inflammatory gene expression in atherosclerotic tissues [109] in rodent models. Fructose-induced proinflammatory effects in adipocytes or in macrophage infiltrating adipose tissue or the liver might be exacerbated by reduced GR signaling and/or enhanced MR signaling [105,110,111], or it may directly stimulate local glucocorticoid activation in these tissues (see below, Section 6). In this regard, the effects of glucocorticoids in the development and progression of T2DM and cardiovascular complications upon the excessive consumption of fructose-containing foods in our modern society need to be further investigated. In line with a role for oxidative stress-related complications, several studies implicated that the administration of antioxidants might prevent the fructose-induced adipose tissue dysfunctions [112], or the progression of steatosis and inflammation in NAFLD [113,114].

To conclude, excess lipid accumulation caused by chronic fructose over-feeding is known to be associated with ER-stress and cellular dysfunction in adipocytes.

5. Role of 11β-HSD1 in Adipocyte Differentiation/Proliferation

A systemic glucocorticoid excess, as observed in Cushing's disease, leads to obesity and all further symptoms of the MetS, with a pathological phenotype of dyslipidemia, insulin resistance, and hypertension [115,116]. However, in abdominally obese patients without Cushing's disease, circulating cortisol levels are not elevated [115]. However, individuals with essential abdominal obesity have an impaired diurnal glucocorticoid rhythm with lower peak levels but higher levels during nadir [117,118]. The total excretion of urinary glucocorticoid metabolites is elevated, probably as a result of an increased hepatic clearance rate due to increased expression of 5α-reductase [21,119–121]. Since circulating glucocorticoid levels are in the normal range, this indicates a higher hypothalamus-pituitary-adrenal (HPA) axis activity. Importantly, the local cortisol synthesis in adipose tissue was found to be increased and is recognized as an important etiologic factor for obesity-related diseases [21,33,122]. Intracellularly, 11β-HSD1 is responsible for the generation of physiologically active glucocorticoids (cortisol, corticosterone) from their inert precursors (cortisone, 11-dehydrocorticosterone), thus regulating glucocorticoid access to glucocorticoid- and mineralocorticoid receptors [28,29,32,34]. It is known that 11β-HSD1 is elevated in adipose tissue in obesity [122,123], where it can contribute to metabolic complications. In contrast, 11β-HSD1 expression remained at normal levels or was found to be reduced in the liver in obesity and T2DM [123–125]. Investigations in transgene mice showed that a moderate overexpression of 11β-HSD1 in adipose tissue was sufficient to induce specific fat accumulation in the VAT [20]. These mice also presented with increased adipocyte size, especially in the VAT, as well as increased non-esterified fatty acid release. Conversely, transgenic 11β-HSD1 KO mice showed reduced hyperglycemia and VAT accumulation and improved insulin sensitivity compared to wild-type mice under conditions of stress and high-fat diet [126].

Earlier, our group reported the expression of hexose-6-phosphate dehydrogenase (H6PDH) in rat epididymal fat, as detected at the level of mRNA, protein, and activity [127]. Adipocytes are

equipped with a functional glucose-6-phosphate transporter (G6PT)—H6PDH—11β-HSD1 system. As exemplified by the model compound metyrapone, an NADPH-depleting agent for modulating local glucocorticoid activation [128], all three components are potential pharmacological targets. Metyrapone administration caused a shift from 11β-HSD1 oxoreductase to dehydrogenase activity in both 3T3-L1-derived and human stem cell-derived differentiated adipocytes [128]. Furthermore, the depletion of luminal pyridine nucleotides in the ER attenuated 11β-HSD1 oxoreductase activity and the decreased accumulation of lipid droplets during preadipocyte differentiation.

During adipocyte maturation, at an early stage, the expression of 11β-HSD1 is low in pre-adipocytes, whereas it increases during the late phase. Earlier studies revealed that glucocorticoids play an important role in preadipocyte differentiation, as active glucocorticoids were required for terminal adipogenesis [129,130] and limit cell proliferation [131]. Inhibition of 11β-HSD1 activity by pharmacological agents or shRNA blocked the capability of inactive 11-oxoglucocorticoids to promote differentiation [132,133]. Thus, these observations emphasize the adipogenic role of glucocorticoids.

6. Effect of Fructose on 11β-HSD1 Expression and Activity

Recent evidence highlights a role of the ER as a nutrient sensor [134], supporting the cellular response to extreme nutritional conditions. The redox state of ER-luminal pyridine nucleotides determines the reaction direction of 11β-HSD1, and alterations of the redox state of pyridine nucleotides are well mirrored by cortisone reduction and cortisol oxidation capacity [29,135]. Over-nutrition with a high sugar load stimulates the local activation of glucocorticoids through the G6PT—H6PDH—11β-HSD1 triad. A previous study addressed the effect of extracellular glucose availability on 11β-HSD1 activity [135]. Lowering glucose concentration in the culture medium caused a decrease in the NADPH/NADP$^+$ ratio, which consequently resulted in a shift from 11β-HSD1 oxoreductase to dehydrogenase activity, thereby lowering the cortisol/cortisone ratio. As reported earlier, at 1 g/L of glucose, 11β-HSD1 oxoreductase activity decreased by 40% compared to cells kept in 4.5 g/L glucose medium. To see whether fructose might have a similar effect, we measured 11β-HSD1 oxoreductase and dehydrogenase activities in cells stably coexpressing 11β-HSD1 and H6PDH (HHH7 clone [136]) with different fructose concentrations in the culture medium (Figure 2). Interestingly, in contrast to glucose, the presence of 1 g/L fructose in the medium as the only carbohydrate source was still capable of maintaining high oxoreductase activity, indicating a high intraluminal NADPH/NADP$^+$ ratio [35]. To extend this dose-dependent effect, we incubated HHH7 cells with various concentrations of fructose. The results showed that even at fructose concentrations as low as 0.1 g/L, efficient 11β-HSD1 oxoreductase activity was observed (50% at 0.1 g/L compared to 4.5 g/L). This suggests that fructose constitutes a more efficient source of ER-luminal NADPH than glucose.

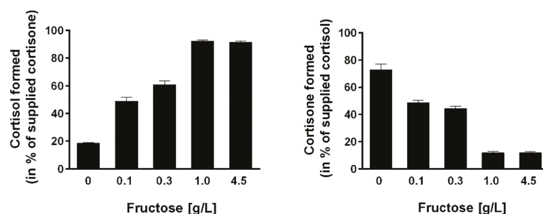

Figure 2. Exogenous fructose regulates 11β-HSD1 activity. Human embryonic kidney cells stably expressing human 11β-HSD1 and H6PDH (HHH7 cell clone [136]) were incubated with different fructose concentrations for 24 h, followed by determination of the 11β-HSD1 oxoreductase (left panel) and dehydrogenase (right panel) activities. Increasing concentrations of extracellular fructose shifted the activity from dehydrogenase to oxoreductase activity. Data represent mean ± S.D. from four independent experiments.

A possible explanation for the fact that fructose seems to be a preferred source for ER-luminal NADPH generation and therefore stimulation of 11β-HSD1-dependent glucocorticoid activation, compared to glucose, might be provided by its intracellular metabolism. Fructose is metabolized in the liver to fructose-1-phosphate, bypassing the key glycolysis regulatory enzyme phosphofructokinase, leading to enhanced lipogenesis. The adipose does not express fructokinase and fructose is converted by hexokinase to F6P [137]. An in vitro study using rat and porcine liver microsomes found that G6P and F6P but not galactose-1-phosphate, glucose-1-phosphate, and fructose-1-phosphate stimulated 11β-HSD1 oxoreductase activity [24]. Interestingly, F6P, unlike G6P, failed to increase 11β-HSD1 oxoreductase activity in porcine adipose microsomes, and the reason for this observation remains unclear. Later, another study using rat liver and adipose microsomes demonstrated that F6P efficiently induced 11β-HSD1 oxoreductase activity. This study also provided evidence for the existence of a F6P transporter in the ER membrane that is distinct of the G6P transporter G6PT, and for the existence of an ER-luminal F6P isomerase, which forms G6P for H6PDH-dependent NADPH generation [25]. Importantly, F6P did not directly serve as a substrate of H6PDH but needed to be first converted to G6P. The luminal F6P isomerase showed different properties than its cytoplasmic counterpart, suggesting that the ER-luminal enzyme is encoded by a different gene. Identification of the gene encoding this ER-luminal F6P isomerase as well as that for the 6-phosphogluconate dehydrogenase (which generates another NADPH molecule in addition to H6PDH) will be important for a better understanding of the coupling of energy status and 11β-HSD1-mediated glucocorticoid activation.

An alternative explanation for the superiority of fructose in ER-luminal NADPH generation can be the preferential transport of fructose and F6P over glucose and G6P through the plasma membrane and ER membrane, respectively. Fructose stimulates its own uptake via GLUT5 at the gene expression level (see above), while the most important glucose transporter in adipocytes, GLUT4, is active only under hyperglycemic conditions. The rates of F6P and G6P transport through the ER-membrane have not yet been compared, an issue that needs to be addressed in future research.

Fructose not only stimulates 11β-HSD1 oxoreductase activity by increasing luminal NADPH generation but also by affecting gene expression. An increased 11β-HSD1 expression and activity was observed in mouse 3T3-L1 adipocytes that were cultivated in medium containing fructose as the only carbohydrate source instead of glucose [35]. As a possible explanation for the elevated expression, an increased ratio of the transcription factors C/EBPα to C/EBPβ, reported earlier to be involved in the transcriptional regulation of 11β-HSD1 [138,139], was detected. Moreover, 3T3-L1 adipocytes differentiated in fructose containing medium had elevated expression of GLUT5, thus further enhancing fructose uptake and stimulating 11β-HSD1 expression and activity. Additionally, lipolysis was induced with increased phosphorylation of perilipin, enhanced expression of hormone sensitive lipase and adipocyte triglyceride lipase, and elevated release of glycerol and FFA. This suggested fructose as a potent adipocyte differentiation stimulant via increasing local glucocorticoid activation.

The above described observations were supported by animal experimentation. A very recent in vivo experiment with Sprague Dawley rats fed with a fructose solution (10% (w/v)) for 9 weeks confirmed our (above established) hypothesis, that fructose overload promotes glucocorticoid production through the enhanced expression and activity of 11β-HSD1 and H6PDH, supplying further NADPH in rat epididymal white adipose tissue [140]. Importantly, these rats developed some of the characteristic features of MetS, such as hypertriglyceridemia and hypertension. Other investigators showed that a shorter exposure of 24 h to a high fructose diet in rats resulted in an elevated expression of 11β-HSD1 in the liver and in VAT [23]. An enhanced expression of 11β-HSD1 as well as GR-regulated lipogenic genes accompanied by an induced adipogenesis was suggested by studies using Wistar rats on 10% fructose in the drinking water for a 9 week period [141–143]. Male rats that were subjected to both fructose-rich diet and chronic unpredictable stress had slightly elevated corticosterone levels, higher 11β-HSD1 expression (but not H6PDH, contrary with the previous animal study), and evidence for increased GR activation [142]. Also, acetyl-CoA-carboxylase, fatty acid synthase and hormone sensitive lipase expression levels were elevated. This may suggest that chronic stress further

exacerbates the fructose-mediated induction of 11β-HSD1 and local glucocorticoid effects on lipolysis and adipogenesis. A recent study with mice that were fed a high-fructose diet for 60 days found elevated glucocorticoid levels in liver and adipose tissue as well as enhanced GR in the nucleus and activation of its target genes [144]. Plasma FFA, TG, insulin, and glucose were increased while hepatic glycogen was decreased. Treatment with the GR antagonist RU486 lowered plasma lipids, tissue glucocorticoids, and GR activation, as well as the expression of its target genes. Additionally, lipid accumulation in adipose tissue decreased and insulin sensitivity was improved. Interestingly, the high-fructose diet resulted in an increased expression of 11β-HSD1, H6PDH, and G6PT in the liver and adipose. Furthermore, the anti-lipogenic transcription factor Hes-1 was down regulated by elevated GR activity while the expression of PPARγ, CD36, and SREBP1-c were enhanced, explaining the elevated FFA and TG production. These observations suggest that high fructose consumption leads to elevated expression and activity of the 11β-HSD1-H6PDH-G6PT triad, promoting local GR activation and glucocorticoid-mediated stimulation of lipolysis and adipogenesis. It will be important to see in follow-on studies whether the selective inhibition of 11β-HSD1 may protect from the adverse metabolic effects of high-fructose consumption.

In this regard, a 16 week treatment of male mice with the American Lifestyle-induced Obesity Syndrome (ALIOS) diet (ad libitum feeding of 45% calories from fat, 11.6% transfats, and 42 g/L high fructose corn syrup (55% fructose, 45% glucose) in the drinking water) recapitulated obesity, insulin resistance, dyslipidemia, and the spectrum of nonalcoholic fatty liver disease (NAFLD) [145]. However, global 11β-HSD1 KO mice were not protected from the metabolic dysregulation following the 16 week ALIOS diet. Glucocorticoids are known to promote steatosis, among other mechanisms by stimulating lipolysis within the adipose tissue, and this leads to increased FFA delivery to the liver, for the production of lipids through increased hepatic de novo lipogenesis [146–148]. 11β-HSD1 KO mice were protected from steatohepatitis upon adding glucocorticoids in the drinking water but as a standard rodent chow [149]. This study also emphasizes the importance of adipose 11β-HSD1 and its impact on the hepatic phenotype. Interestingly, the ALIOS diet led to an early transition to hepatic inflammatory disease with elevated markers of inflammation, immune cell infiltration, and fibrosis in 11β-HSD1 KO mice, indicating a transition to non-alcoholic steatohepatitis (NASH) [145]. Why the global 11β-HSD1 KO mice were not protected against the ALIOS diet in the study by Larner et al. [145], but 11β-HSD1 KO mice were resistant against hyperglycemia induced by obesity or stress in the study by Kotelevtsev et al. [126], remains unclear. Analysis of the differences in animal maintenance, diet, and treatment duration may provide an explanation. It needs to be noted that the life-long adaptation by compensatory mechanisms including an elevated adrenal glucocorticoid production may be responsible for the lack of protection from the high fructose, high transfat diet in 11β-HSD1 KO mice, and that the administration of pharmacological inhibitors, especially when targeted to the adipose tissue, may lead to a different outcome.

7. Conclusions

Both excessive fructose consumption and increased intracellular glucocorticoid activation have been suggested to contribute to the pathogenesis of the MetS (Figure 3). Fructose is suggested to be the most hypertriglyceridemic sugar. However, it is important to investigate whether abdominal obesity exacerbates the hypertriglyceridemic effect of the high fructose diet and whether increased glucocorticoids further aggravate the adverse metabolic effects of high fructose. Independently of the consumed fructose, elevated glucocorticoids and central obesity, especially visceral obesity, are associated with a higher risk for T2DM and MetS. Therefore, future investigations on the effects of fructose should consider the source and dietary form of fructose (solid food or beverage) and should include careful controls regarding the sex-, genetics-, stress-, and obesity-related differences in responsiveness.

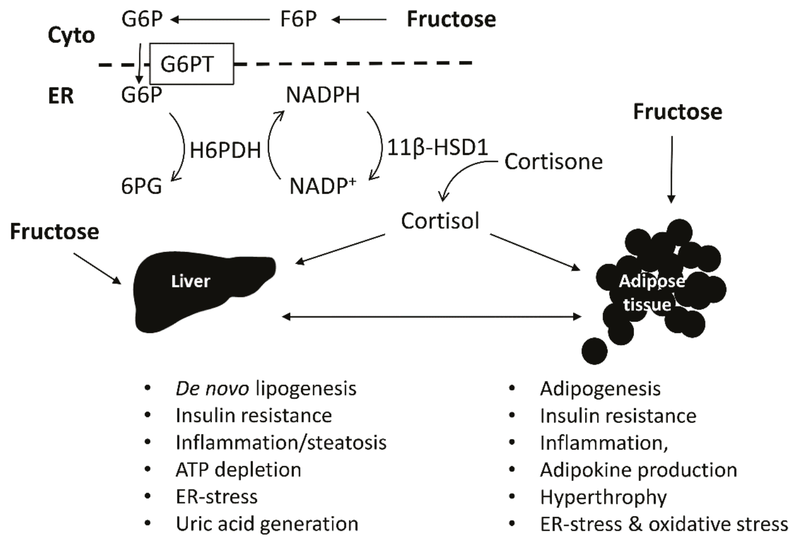

Figure 3. Effect of fructose on liver and adipose tissue, their interconnections, and impact of glucocorticoid activation. Excessive fructose consumption is thought to be associated with hepatic steatosis, cellular stress, and inflammation of the MetS. The enhanced glucocorticoid production also has a crucial role in the regulation of adipocyte differentiation and cellular metabolism. 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; 6PG, 6-phosphogluconate; Cyto, cytoplasm; ER, endoplasmic reticulum; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G6PT, glucose-6-phosphate transporter in the ER-membrane; H6PDH, hexose-6-phosphate dehydrogenase

The importance of the G6PT-H6PDH-11β-HSD1 system in the ER-lumen received a distinct focus in the past few years, providing a novel pharmaceutical potential to intervene in the progression of MetS and prevent its diabetic and cardiovascular consequences. However, there are important questions remaining. Whether or not pharmacological inhibition of H6PDH or G6PT may offer therapeutic benefits remains fully unexplored and further basic research to better understand the functions of these two proteins is needed. The fact that global 11β-HSD1 KO mice, which are subjected to adaptation by life-long compensatory mechanisms, were not protected against the ALIOS diet in the study by Larner et al. [145] is an argument against protection from adverse metabolic effects of high fructose containing diet by the pharmacological inhibition of 11β-HSD1. However, selective inhibition of 11β-HSD1 in adipose tissue might be superior to global enzyme deficiency due to more pronounced feedback regulation and increased adrenal glucocorticoid production in the latter situation. Thus, the effect of selective inhibition of 11β-HSD1 specifically in adipose tissue should be investigated. Furthermore, species-specific differences need to be considered. Fructose as well as glucocorticoid metabolism in rodents and human are different in several aspects, and, ideally, clinical studies should be performed to better understand the link between high fructose intake and glucocorticoid action.

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

Abbreviations

AMPK: Adenosine 5′-monophosphate (AMP)-activated protein kinase; ATP: Adenosine Triphosphate; CVD: Cardiovascular Disease; CHOP: CCAAT-enhancer-binding protein homologous protein; ER: endoplasmic reticulum; F1P: fructose-1-phosphate; G6P: glucose-6-phosphate; G6PT: G6P translocase; GLUT5: glucose transporter 5; Fru-1-P: Fructose-1-phophate; FFA: free fatty acid; H6PD: hexose-6-phosphate dehydrogenase; HFD: high fat diet; 11β-HSD1: 11β-hydroxysteroid dehydrogenase type 1; MetS: metabolic syndrome; NADPH: reduced nicotinamide adenine dinucleotide phosphate; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; PPARα: Peroxisome Proliferator-Activated Receptor α; ROS: reactive oxygen species; SREBP1c: Sterol Response Element Binding Protein 1c; T2DM: type II diabetes mellitus; TG: triglycerides; TNF: tumor necrosis factor; UPR: unfolded protein response; VAT: visceral adipose tissue; VLDL: Very Low Density Lipoprotein; XBP-1: X-box binding protein 1.

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Deleterious Metabolic Effects of High Fructose Intake: The Preventive Effect of *Lactobacillus kefiri* Administration

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Abstract: Modern lifestyle and diets have been associated with metabolic disorders and an imbalance in the normal gut microbiota. Probiotics are widely known for their health beneficial properties targeting the gut microbial ecosystem. The aim of our study was to evaluate the preventive effect of *Lactobacillus kefiri* (*L. kefiri*) administration in a fructose-rich diet (FRD) mice model. Mice were provided with tap water or fructose-added (20% *w*/*v*) drinking water supplemented or not with *L. kefiri*. Results showed that probiotic administration prevented weight gain and epidydimal adipose tissue (EAT) expansion, with partial reversion of the adipocyte hypertrophy developed by FRD. Moreover, the probiotic prevented the increase of plasma triglycerides and leptin, together with the liver triglyceride content. Leptin adipocyte secretion was also improved by *L. kefiri*, being able to respond to an insulin stimulus. Glucose intolerance was partially prevented by *L. kefiri* treatment (GTT) and local inflammation (TNFα; IL1β; IL6 and INFγ) was completely inhibited in EAT. *L. kefiri* supplementation generated an impact on gut microbiota composition, changing *Bacteroidetes* and *Firmicutes* profiles. Overall, our results indicate that the administration of probiotics prevents the deleterious effects of FRD intake and should therefore be promoted to improve metabolic disorders.

Keywords: gut microbiota; fructose-rich diet; adipose tissue; probiotics

1. Introduction

Obesity has been defined by the World Health Organization as an Adipose Tissue (AT) excess that could be harmful to the organism, predisposing to pathologies such as type II Diabetes Mellitus, cardiovascular disease, dyslipidemias, fatty liver disease, and certain cancers. AT mass expansion is associated with serious changes in AT architecture and function, among which adipocyte hypertrophy is one of the most relevant features. Hypertrophic adipocytes are characterized by releasing high amounts of leptin, pro-inflammatory cytokines, low adiponectin, and insulin-resistance [1,2]. Furthermore, AT mass expansion induces a shift from an anti- to pro-inflammatory profile of immune cells resident in this tissue, leading to a general pro-inflammatory state of AT [3,4].

Obesity is a multifactorial disorder caused by the interaction of genetic background and environmental factors, such as altered eating habits [5]. Modern diets are characterized by high carbohydrate intake, especially fructose-sweetened beverages, and have been associated with high prevalence of overweight and Metabolic Syndrome (MS) in humans [6]. High-fructose feeding has

been widely used in animal models to induce obesity and MS phenotype [7–10]. In the present study, we used a fructose-rich diet intake (FRD, 20% *w*/*v* in drinking water), which is far from fructose intake by humans, to generate a mice model of obesity. Previously, FRD has been related to the development of insulin resistance, dyslipidemias, increased abdominal AT mass, and changes in the pattern of AT adipokine secretion [7,8,11]. Partly, these metabolic disorders are a consequence of fructose-induced hepatic de novo lipogenesis, and the resulting increase in AT fatty acid uptake [12,13].

Gut microbiota is composed of 1 to 10 trillion microorganisms, mainly bacteria, among which approximately 90% belong to the *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria* phyla [14]. Symbiotic relationships between bacteria and their hosts modulate several physiological processes such as nutrients uptake, metabolism, and immune response, among others [15]. Environmental factors such as diet, treatment with antibiotics, and exercise can modulate gut microbiota composition. Obesity has been associated with gut microbiota dysbiosis, contributing to the establishment of characteristic alterations related to obesity. Since transplantation of lean gut microbiota to obese mice can rescue the obese phenotype [16], strategies to manipulate the composition of the gut microbiota have gained considerable importance for metabolic pathologies management. Probiotics are defined as live microorganisms that, when administered, exert positive health effects in the host. It is largely accepted that probiotics are involved in the maintenance of healthy gut microbiota, and for this reason their use has emerged as a potential therapy against MS and obesity [17].

Kefir is a food product obtained by fermentation of milk with "kefir grains". These grains are constituted by a complex symbiotic microbiota, mainly of yeast, lactic acid, and acetic acid bacteria confined in a matrix of polysaccharides and proteins [18,19]. Several health-promoting properties have been associated with kefir consumption [19,20], and the study of the beneficial properties of kefir-isolated microorganisms can be considered as a very important field for the development of functional foods. *Lactobacillus kefiri* is one of the most important lactobacilli obtained from kefir grains [21,22]. Different in vitro studies have revealed that secretion products and surface proteins from different *L. kefiri* strains can exert a protective action against intestinal pathogens such as *Salmonella enterica* [23] and *Clostridium difficile* [24]. Moreover, Carey and Kostrzynska reported that *L. kefiri* attenuates the pro-inflammatory response in intestinal epithelial cells induced by *Salmonella typhimurium*, and Hong et al. showed its influence on Th1 and pro-inflammatory cytokine production in macrophages [25,26].

Recent studies have demonstrated that *L. kefiri* CIDCA 8348 strain resists passage through simulated gastrointestinal conditions [27] and its oral administration is safe to mice [28]. Interestingly, mice treated with *L. kefiri* CIDCA 8348 showed a down-regulation of the gene expression of pro-inflammatory mediators and an up-regulation of anti-inflammatory molecules, secreted IgA and mucins in the gut [29]. Based on this evidence, we aimed to evaluate the preventive effect of *L. kefiri* CIDCA 8348 administration on the metabolic alterations caused by FRD in mice.

2. Material and Methods

2.1. Bacterial Strain and Growth Conditions

A kefir-isolated *Lactobacillus kefiri* strain (CIDCA 8348, *L. kefiri*) [30] from the collection of the "Centro de Investigación y Desarrollo en Criotecnológico de Alimentos" (La Plata, Argentina) was used for experiments. The strain was cultured in MRS-broth (DIFCO, Detroit, MI, USA) at 37 °C for 48 h under aerobic conditions. Frozen stock cultures were stored at −80 °C in skim milk until use.

2.2. Animals and Treatment

Normal adult male Swiss mice (four months of age, n = 15 mice per group) were kept in a temperature-controlled environment (20–22 °C and fixed 12 h light/12 h dark cycle, lights on at 07:00 a.m.) and fed *ad libitum* with Purina commercial rat chow. Mice were divided into two groups: one was provided with tap water and the other with a 20% fructose solution (*w*/*v*, Sigma-Aldrich,

St. Louis, MO, USA) added to tap water for 6 weeks (conventionally called fructose rich diet, FRD). Each group was randomly divided and administered *L. kefiri* (10^8 CFU dissolved in milk; CTR-Lk and DRF-Lk groups) or milk alone (CTR and FRD groups) by oral gavage every 48 h during the 6-week diet. Food intake and body weight were measured every 48 h. On experimental day, mice were euthanized under non-fasting conditions (between 08:00 a.m. and 09:00 a.m.) and trunk blood was collected; plasma samples were then frozen (−20 °C) until metabolite measurements (Section 2.3). Inguinal AT (IAT, subcutaneous depot), Epididymal AT (EAT, visceral depot) and Retroperitoneal AT (RPAT, visceral depot) were aseptically dissected and weighed. EAT was kept in sterile Dulbecco's Modified Eagle's Medium-Low Glucose (1 g/L) (DMEM-LG) for further procedures. Animals were euthanized according to protocols for animal use, in agreement with National Institutes of Health (NIH) guidelines for the care and use of experimental animals. All experiments were approved by our Institutional Animal Care Committee (approval code 020916).

2.3. Peripheral Metabolite Measurements

Non-fasting plasma levels of leptin (LEP, n = 15) were determined by specific radioimmunoassays (RIAs) previously developed in our laboratory [31]. Non-fasting plasma levels of glucose (Glu, n = 15) and triglycerides (Tg, n = 15) were measured using commercial kits (Wiener Lab., Rosario, Argentina).

2.4. EAT Adipocyte Isolation and Incubation

Fresh EAT pads were dissected, weighed and digested with collagenase as previously reported [32]. Briefly, fat tissue was minced and digested using 1 mg/mL collagenase solution in DMEM (at 37 °C, for 1 h). After centrifugation (1000 rpm for 15 min), floating mature adipocytes were separated and diluted up to a density of approximately 200,000 cells per 900 µL DMEM-1% BSA. Adipocytes were distributed in 24 multi-well plates and incubated for 45 min at 37 °C in a 5% CO_2 atmosphere with medium alone (basal) or medium containing 10 nM insulin (Novo Nordisk Pharma AG, Küsnacht, Switzerland) [33]. After incubation, medium was carefully aspirated and kept frozen (−20 °C) until measurement of LEP concentrations as described above (n = 6 independent experiments).

2.5. EAT Pad Histology

For histological studies, freshly dissected EAT pads (n = 4 per group) were fixed in 4% paraformaldehyde, then washed with tap water, immersed in a series of graded ethanol solutions (70%, 96% and 100%), and clarified in xylene before paraffin embedding [34]. Four-micrometer sections were taken from different levels of the blocks and stained with hematoxylin-eosin. Quantitative morphometric analysis was performed using a RGB CCD Sony camera and Image Pro-Plus 4.0 software (Image ProPlus6.0, Rockville, MD, USA). For each tissue sample, seven sections and three levels were selected. Systematic random sampling was used to select 15 fields for each section (magnification, ×400) and 2500 cells per group were examined. Adipocyte area was measured.

2.6. Glucose Tolerance Test (GTT)

Four days before the end of the protocol, six mice from each experimental group were fasted for 10 h (from 10:00 p.m. to 8:00 a.m.) and then glucose (2 mg/kg BW) was administered via intraperitoneal (IP) injection. Blood was collected by the tail cut method. Glucose was measured at 0, 30, 60 and 120 min after glucose challenge by one-touch glucometer (Accu-Chek Performa, Roche, Mannheim, Germany). Area under the curve was calculated using Graph Pad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

2.7. Liver Lipid Content

Fifty mg of the liver (n = 6 per group) was homogenized in a 5% solution of 500 μL Triton X-100 in phosphate-buffered saline (PBS). The homogenate was incubated at 80–100 °C for 5 min and centrifuged at 10,000× g for 10 min. Triglyceride (Tg) was measured in the supernatants using a commercial kit (Wiener Lab, Rosario, Argentina).

2.8. RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA from EAT (n = 6 per group) was isolated by the Trizol extraction method (Invitrogen, Life Tech., Carlsbad, CA, USA) and reverse-transcribed using random primers (250 ng) and RevertAid Reverse Transcriptase (200 U/μL, Thermo Scientific, Vilnius, Lithuania). Two μL cDNA were amplified with HOT FIRE Pol EvaGreenqPCR Mix Plus (Solis BioDyne, Tartu, Estonia) containing 0.5 μM of each specific primer, using a Rotor Gene Q (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) efficiency was near 1. Expression levels were analyzed for β-actin (ACTβ, reporter gene), Adiponectin (Adipo), Leptin (Ob), Lipoprotein Lipase (LPL), Fatty Acid Synthase (FAS), Hormone Sensitive Lipase (HSL), Adipose Triglyceride Lipase (ATGL), Tumor Necrosis Factor α (TNFα), Interleukin 1β (IL1β), Interleukin 6 (IL6), and Interferon γ (IFNγ). Designed primers are shown in Table 1. Relative changes in the expression level of one specific gene ($\Delta\Delta C_t$) were calculated by the ΔC_t method.

Table 1. Primers used for real time PCR analysis.

Gene	Sequence (5′-3′)	GBAN	Size Product (bp)
ACTβ	Fw: TTTGCAGCTCCTTCGTTGCC Rv: ACCCATTCCCACCATCACAC	NM_007393.5	189
Ob	Fw: ACCAGGATCAATGACATTTCACAC Rv: GGCTGGTGAGGACCTGTTGA	NM_008493.3	148
Adipo	Fw: GGAACTTGTGCAGGTTGGATG Rv: CCCTTCAGCTCCTGTCATTCC	NM_009605.5	171
LPL	Fw: AGGACCCCTGAAGACAC Rv: GGCACCCAACTCTCATA	NM_008509.2	149
ATGL	Fw: CCACTCACATCTACGGAGCC Rv: AATCAGCAGGCAGGGTCTTC	NM_001163689.1	198
HSL	Fw: AGTTACCATCTCACCTCC Rv: CTTGCTGTCCTGTCCTTC	NM_010719.5	94
FAS	Fw: CAAGCAGGCACACACAATGG Rv: GCCTCGGAACCACTCACA	NM_007988.3	141
TNFα	Fw: CATCTTCTCAAAATTCGAGTGACAA Rv: CCTCCACTTGGTGGTTTGCT	NM_013693.3	63
IFNγ	Fw: TGGCATAGATGTGGAAGAAAAGAG Rv: TGCAGGATTTTCATGTCACCAT	NM_008337.4	81
IL1β	Fw: CTTGTGCAAGTGTCTGAA Rv: AGGTCAAAGGTTTGGAAG	NM_008361.4	143
IL6	Fw: GTTCTCTG GAAATCGTGGAAA Rv: AAGTGCATCATCGTTGTTCATACA	NM_031168.2	77

Specific primers used for real time PCR analyses; Fw: Forward, Rv: Reverse; GBAN: GenBank Accession Number; bp: base pairs.

2.9. Leptin Measurement

Medium LEP concentration was determined by specific RIA [35]. In this assay, the standard curve ranged between 50 and 12,500 pg/mL, with intra- and inter-assay variation coefficients of 4–6% and 5–8%, respectively.

2.10. Microbiota Analysis in Feces

Fecal samples were collected at the end of the experimental protocol and were stored at −80 °C prior to use for microbiota analysis. DNA extraction was performed using the *AccuPrep* Stool DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions.

2.10.1. Quantitative PCR of Microbiota Populations

Quantification of bacterial populations was carried out using primers synthesized by Genbiotech (Buenos Aires, Argentina). Primer sequences were previously described [29,36]. PCR reactions were performed in a Rotor Gene Q (Qiagen, Hilden, Germany) using HOT FIRE Pol EvaGreenqPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Twenty ng DNA and 0.2 μmol L^{-1} of each primer were used in PCR mix. A negative control reaction without template was included for each primer combination. Melting curve was conducted from 70 °C to 90 °C, read every 0.5 °C during 2 s. For standard curves we used PCR products generated from a pool of purified genomic DNA from the different samples and the primers previously described [29,36]. Results were expressed as number of copies/g wet weight feces.

2.10.2. Qualitative Analysis by PCR-DGGE

Primers 518r (5′-ATTACCGCGGCTGCTGG-3′) and 338f (5′-CTCCTACGGGAGGCAGCAG-3′) coupled to a 50-GC clamp [37], targeting the V3 region of the 16S rRNA subunit [38], were used to assess microbial diversity in each sample. PCR was performed in a Stratagene Gradient Cycler (Agilent Technologies Inc., Philadelphia, PA, USA) using 1U of PFU DNA Polymerase (PB-L, EmbioTec SRL, Buenos Aires, Argentina) per 50 pg of DNA template. The PCR products were separated in 8% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) with a range of 40–60% denaturing gradient (100% denaturant consisted of 7 M urea and 40% deionized formamide) cast in a DDGE-2401 device (C.B.S Scientific Co., Del Mar, CA, USA). The electrophoresis was performed in TAE 0.5X buffer for 16 h at a constant voltage of 100 v and a temperature of 60 °C. Gels were stained with SYBR Gold 0.01 μL/mL (Invitrogen, Life Technologies, Carlsbad, CA, USA) in TAE 1X buffer and visualized in a Bio-Rad Universal Hood II gel documentation system (Bio-Rad laboratories Inc., Hercules, CA, USA). PyElph 1.4 software was used to calculate the dendrograms using the UPGMA (unweighted pair group method with arithmetic mean clustering algorithm) [39].

2.11. Statistical Analysis

Results are expressed as mean values ± S.E.M. Data were analyzed by ANOVA (one-way) method followed by Fisher´s test. Body weight data were analyzed using a multivariate test (IBM SPSS statistics 22, IBM Corp., New York, NY, USA). To determine the differential effect of the treatments, ANOVA (two-way) analysis was performed followed by Tukey's test. p values lower than 0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. L. kefiri Administration Prevents Body Weight Gain and AT Expansion

Caloric intake for FRD mice was higher than that for CTR mice (Figure 1A; $P < 0.05$) and, as expected, was accompanied by an increase in body weight (Figure 1B; $P < 0.05$ vs. CTR). *L. kefiri* administration to CTR mice (CTR-Lk group) did not modify caloric intake or body weight compared to CTR animals. However, administration of *L. kefiri* during FRD intake prevented the increase in body weight without changing the caloric intake ($P < 0.05$ vs. FRD). Multivariate analysis showed an interaction between diet and probiotic administration had a significant effect ($P = 0.02$). FRD consumption has been widely used in animal models to induce metabolic disorders as those observed

in human MS; one of these features is the visceral AT expansion. Our results showed that FRD induced a significant increase in EAT mass ($P < 0.05$). Interestingly, probiotic treatment inhibited EAT mass expansion induced by FRD intake (FRD-Lk mice; $P < 0.05$) and also decreased EAT mass in CTR-Lk mice compared to CTR (Figure 1C; $P < 0.05$). With regard to other AT depots studied, although no significant differences were found among experimental groups, FRD mice showed a trend toward increased IAT and RPAT mass, which was not observed in FRD-Lk mice (Figure 1D,E).

Unhealthy AT expansion has been associated with hypertrophic adipocytes while hyperplastic AT expansion prevents AT dysfunctions. Histological analysis of adipocyte size from EAT showed that *L. kefiri* administration did not affect cell size in CTR-Lk mice (Figure 1F). On the other hand, FRD induced an increase in adipocyte size ($P < 0.01$) that was not observed in FRD-Lk adipocytes. Thus, high fructose consumption generated an unhealthy EAT expansion but was partially prevented by the probiotic treatment.

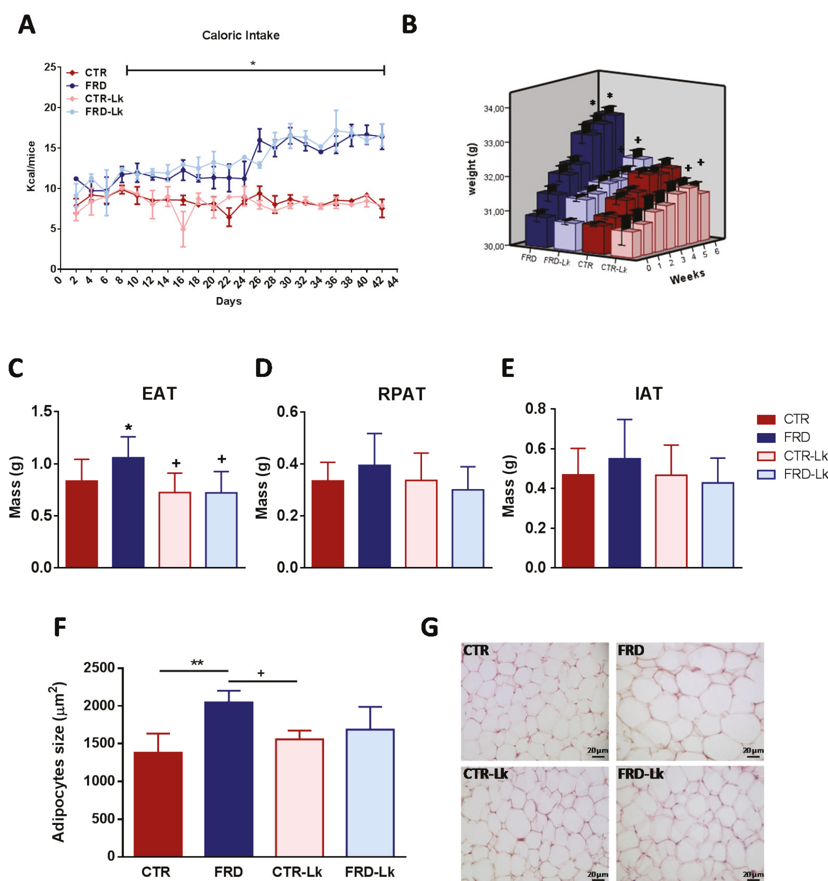

Figure 1. Mean of caloric intake, body weight and AT expansion. (**A**) Caloric intake (* $P < 0.05$ vs. CTR and CTR-Lk) and (**B**) body weight from the different groups. (* $P < 0.05$ vs. CTR. $^{+}$ $P < 0.05$ vs. FRD) (**C**) EAT, (**D**) RPAT and (**E**) IAT mass were measured. * $P < 0.05$ vs. CTR and $^{+}$ $P < 0.05$ vs. FRD. ($n = 15$ mice per group). (**F**) EAT adipocyte size. ($n = 4$ mice per group). ** $P < 0.001$ vs. CTR and $^{+}$ $P < 0.05$ vs. FRD. (**G**) Representative EAT histological samples stained with hematoxylin-eosin. Values are means ± SEM.

3.2. Metabolic Alterations and Glucose Homeostasis Impairment Were Improved by Probiotic Treatment

FRD mice showed an impaired metabolic profile, characterized by higher plasmatic concentration of Tg and LEP than CTR mice, without changes in Glu plasmatic levels (Figure 2A–C; $P < 0.05$ and $P < 0.01$, respectively). *L. kefiri* treatment did not modify these parameters in CTR-Lk mice. Interestingly, FRD-Lk mice showed circulating levels of Tg and LEP similar to CTR mice, which reveals the beneficial effect of *L. kefiri* administration on the metabolic profile from high-fructose feeding mice.

Additionally, liver lipid content was similar for CTR and CTR-Lk mice (Figure 2D). As expected, considering the strong lipogenic capacity of fructose, FRD mice showed higher liver lipid levels ($P < 0.05$). This increase was in part attenuated by the administration of *L. kefiri* (FRD-Lk group).

GTT was performed to assess glucose homeostasis in the different groups. Administration of *L. kefiri* to CTR mice did not modify GTT response, as shown in Figure 2E. For the case of FRD group, we observed an impaired glucose tolerance, which was clearly evidenced in a higher area under the curve (Figure 2F; $P < 0.05$). This glucose intolerance was partially prevented by *L. kefiri* treatment (FRD-Lk).

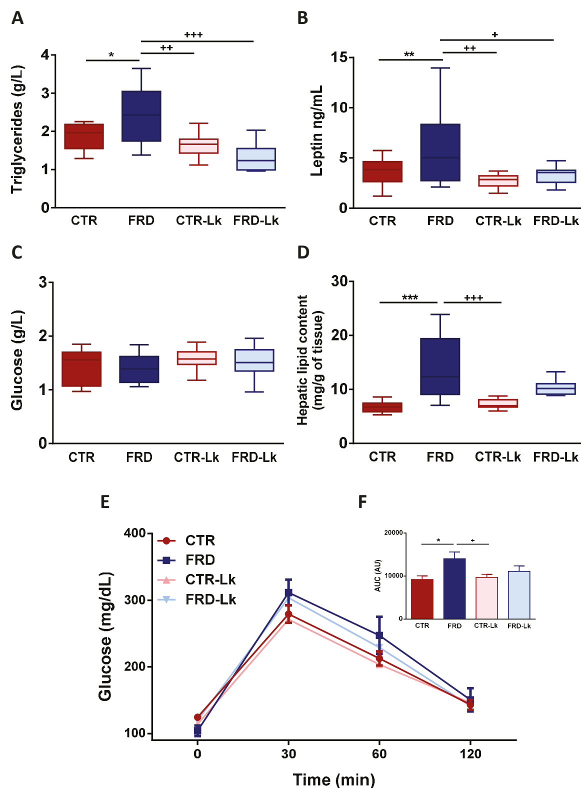

Figure 2. Metabolic Parameters and Glucose Tolerance Test. (**A**) Triglycerides, (**B**) Leptin and (**C**) Glucose plasma concentrations were recorded. (n = 15 mice per group). (**D**) Liver triglycerides content was assessed in the four groups. Values are means ± range (n = 6 mice per group). (**E**) GTT was performed four days before to the end of the protocol. Glucose concentration was measured at 0, 30, 60 and 120 min after glucose challenge. (**F**) Area under the curve was calculated. Values are means ± SEM (n = 6 mice per group). * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ vs. CTR. $^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$ and $^{+++}$ $P < 0.001$ vs. FRD.

3.3. *L. kefiri* Administration Reduces EAT Dysfunctions Induced by FRD

AT dysfunction directly correlates with adipocyte size. Hypertrophic adipocytes are insulin resistant and secrete an altered adipokyne pattern (LEP and Adipo) and pro-inflammatory cytokines. As shown in Figure 3A,B, isolated EAT adipocytes from CTR-Lk secreted similar amount of LEP as CTR mice, both spontaneously and after insulin stimulation. FRD hypertrophic adipocytes secreted more LEP than CTR ones, and did not respond to insulin stimulus ($P < 0.0001$ and $P < 0.0001$ vs. CTR, respectively). Under basal condition, adipocytes from FRD-Lk showed an intermediate secretion of LEP between CTR and FRD adipocytes, suggesting a partial protection exerted by *L. kefiri* administration on the impairment of LEP secretion caused by FRD. Interestingly, when FRD-Lk adipocytes were insulin-stimulated, they significantly increased their LEP release ($P < 0.001$ vs. FRD-Lk basal), indicating insulin sensitivity recovery ($P < 0.05$ vs. FRD).

When mRNA expression was analyzed, EAT from FRD expressed significantly higher LEP levels than CTR adipocytes (Figure 3C; $P < 0.01$), while *L. kefiri* administration protected FRD-Lk mice from this increase (Figure 3C; $P < 0.001$ vs. FRD), in agreement with results detailed above. Neither FRD nor *L. kefiri* treatment altered adiponectin mRNA levels. To assess the status of lipid metabolism in EAT we evaluated the expression of different enzymes involved in lipolysis/lipogenesis pathway. As shown in Figure 3D, EAT from FRD mice expressed higher mRNA levels of HSL and LPL than CTR (HSL: $P < 0.05$ and LPL: $P < 0.05$) and CTR-Lk mice (HSL: $P < 0.05$ and LPL: $P < 0.01$), whereas Lk administration to FRD mice prevented this increase (HSL: $P < 0.05$ and LPL: $P < 0.01$ vs. FRD).

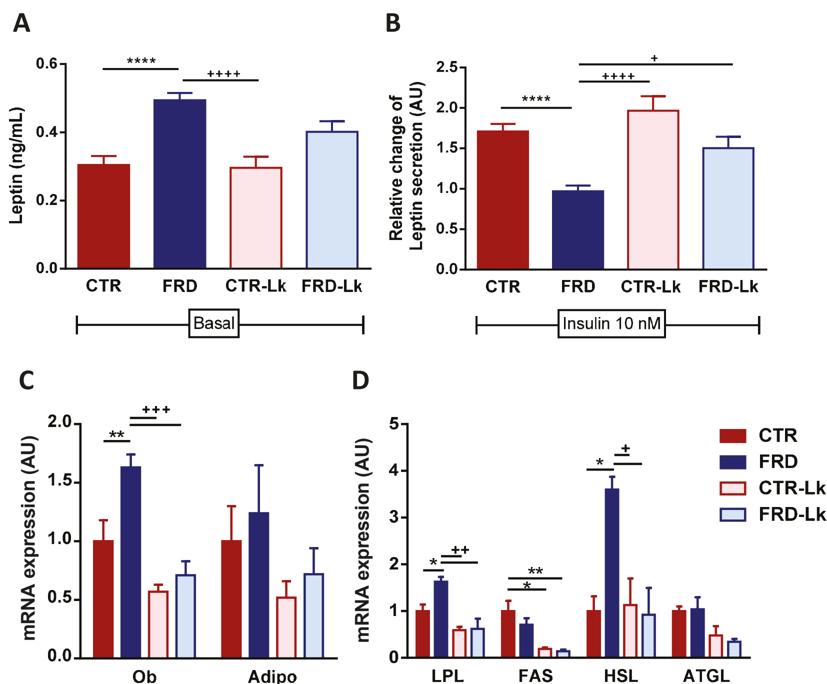

Figure 3. Improvement of EAT function. (**A**) Leptin secretion from cultured adipocytes in basal conditions. (**B**) Relative change of leptin secretion after insulin stimulus compared to basal secretion for each group. ($n = 6$ independent experiments). EAT mRNA expression of (**C**) LEP (Ob) and adiponectin (Adipo) and (**D**) lipid metabolism-related enzymes (LPL, HSL, ATGL, FAS). ($n = 6$ mice per group). Values are means ± SEM. * $P < 0.05$; ** $P < 0.01$ and **** $P < 0.0001$ vs. CTR. $^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$; $^{+++}$ $P < 0.001$ and $^{++++}$ $P < 0.0001$ vs. FRD.

3.4. *L. kefiri* Treatment Protects EAT from Inflammation Induced by FRD

It is well known that obesity is associated with AT chronic inflammatory state caused by the change of AT resident immune cells from anti-inflammatory Type 2 to pro-inflammatory Type 1, favoring insulin-resistance. In this regard, our results showed that FRD intake induced an inflammatory state in EAT, evidenced by significant increase of IL6, IL1β, TNFα and IFNγ expression (Figure 4; $P < 0.05$ vs. CTR), that was prevented by *L. kefiri* treatment (IFNγ: $P < 0.05$; IL1β: $P < 0.01$ and IL6: $P < 0.01$ vs. FRD). Noticeably, we found that *L. kefiri* administration did not modify mRNA levels of pro-inflammatory cytokines in EAT from CTR-Lk mice.

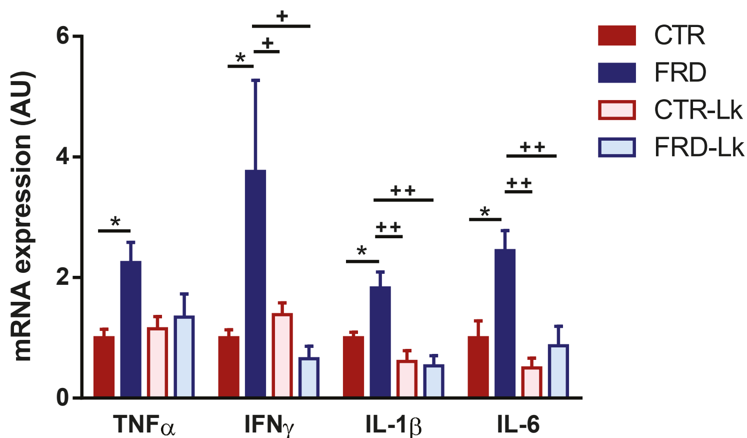

Figure 4. Inflammatory profile of EAT. Gene expression of pro-inflammatory cytokines (TNFα, INFγ, IL1β, IL6) in EAT from the four experimental groups. Values are means ± SEM ($n = 6$ mice per group). * $P < 0.05$ vs. CTR. $^{+}$ $P < 0.05$ and $^{++}$ $P < 0.01$ vs. FRD.

3.5. Effects of FRD and *L. kefiri* Administration on Gut Microbiota Composition

To study the effects of diet and probiotic administration on gut microbiota structure, we analyzed the fecal bacterial composition by PCR-DGGE and qPCR. Both FRD and Lk administration produced qualitative changes in the microbial community composition, since the cluster analysis based on the Pearson product-moment correlation coefficient and UPGMA linkage allowed differentiation of the experimental groups in separated clusters (Figure 5), without differences in the number of amplification bands generated from each sample (not shown). Regarding qPCR assays, we performed two-way ANOVA analysis to determine if FRD or Lk or the interaction of both variables have some effect on fecal bacterial amounts. Firstly, no differences in the total number of bacteria between groups were found, although a trend to increase was observed in mice under probiotic treatment, independently of diet intake (Table 2). When specific bacterial phyla were analyzed, we found a different effect regarding diet and probiotic administration. FRD feeding decreased the *Lactobacillus* spp. (Figure 6A, $P = 0.015$) and increased *Bacteroides fragilis* quantities independently of probiotic administration (Figure 6B, $P = 0.0074$). On the other hand, when *L. kefiri* administration was analyzed we found an increase in *Firmicutes* and *Bacteroidetes* phyla (Figure 6A, $P = 0.007$ and $P = 0.027$ respectively), and in *Lactobacillus murinus* and *Bacteroides fragilis* species (Figure 6B, $P = 0.0048$ and $P = 0.0072$ respectively), in spite of mice receiving or not the FRD. No differences were found in *L. acidophilus* group when both variables were analyzed.

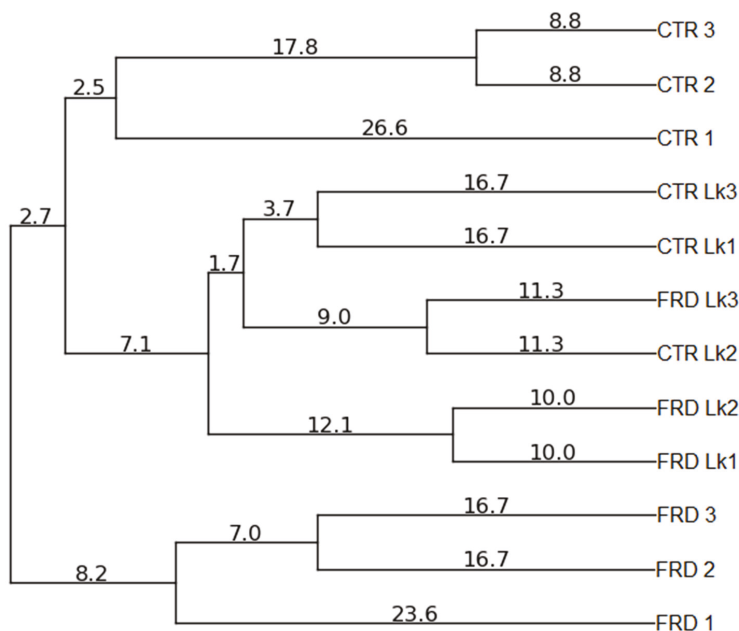

Figure 5. Qualitative analysis of fecal microbiota profile. Dendrogram for the total bacterial DGGE profiles. Clustering analysis was performed using the UPGMA linkage.

Table 2. Total bacteria quantification in feces.

	CTR	FRD	CTR-Lk	FRD-Lk
Total Bacteria (*N* of copies/g of feces)	$3.73 \times 10^9 \pm 5.6 \times 10^8$	$4.97 \times 10^9 \pm 5.0 \times 10^8$	$5.94 \times 10^9 \pm 6.7 \times 10^8$	$6.97 \times 10^9 \pm 7.8 \times 10^8$

After the overall variable analyses, we compared the microbiota composition among the different groups. Interestingly, we found that *Bacteroidetes* population from FRD-Lk mice was significantly increased compared to CTR ($P < 0.05$). Accordingly, *Bacteroides fragilis* (*Bacteroidetes* phylum) was also increased in FRD-Lk ($P < 0.05$ vs. CTR and FRD) and in CTR-Lk mice ($P < 0.05$ vs. CTR). Additionally, *Lactobacillus murinus* was also more abundant in FRD-Lk and in CTR-Lk ($P < 0.05$) than their counterparts without probiotic administration (Figure 6B). These changes in the microbiota composition in Lk-treated mice could suggest the presence of a healthier microbiota, which could be related to the beneficial metabolic changes found in them.

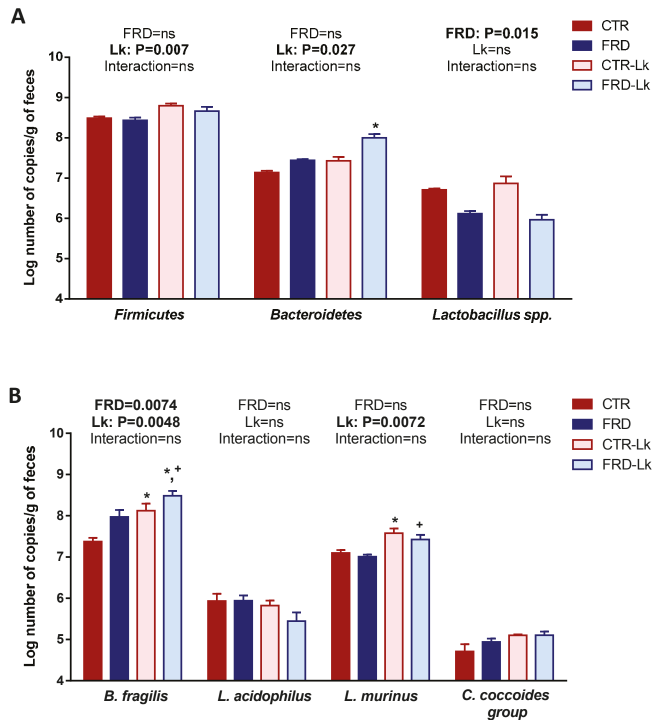

Figure 6. Analysis of microbiota composition in feces. (**A**) Lactobacillus spp., Lactobacillus acidophilus (*L. acidophilus*) and Lactobacillus murinus (*L. murinus*) quantification. (**B**) Firmicutes and Bacteroidetes phyla, Clostridium coccoides group (*C. coccoides* group) and Bacteroides fragilis (*B. fragilis*) quantification. Two-way ANOVA was performed for variable analysis and Tukey's multiple comparisons post-test was performed for group-to-group comparisons. ns = no significant differences. Values are means ± SEM (n = 3 mice per group). * $P < 0.05$ vs. CTR, $^{+}$ $P < 0.05$ vs. FRD.

4. Discussion

Over the few last decades, the importance of the symbiotic relationship between gut microbiota and host in energy absorption, immune system and metabolism has been described [40]. Alterations caused by environmental factors on gut microbiota composition could lead to host metabolic disorders, as has been observed in both obese humans and rodents [41–43]. Since probiotics modulate gut microbiota and also affect host metabolism, the use of probiotics has been associated with several metabolic improvements in obese phenotypes [40,44]. In this regard, several studies have demonstrated the benefits of the use of lactobacilli as probiotics, improving liver pathologies, among others [45,46].

Fructose-sweetened beverages are one of the most remarkable components of modern diets and their consumption has increased notably in the last few decades [6]. Although diet is one factor that can affect gut bacterial profile in early life, as well as in adulthood, the effect of fructose on gut microbiota has been poorly studied. In fact, the use of probiotics as a preventive tool has been addressed mostly in high-fat diet models. FRD has been widely used to induce MS and obesity in animal models [7,8,11]. Our current results show that FRD intake for six weeks was effective in inducing an increase in body weight and EAT mass. These changes were accompanied by higher Tg and LEP plasma levels, peripheral insulin-resistance and increased liver lipid content, confirming the deleterious effects caused by this diet.

In the present study, we proposed to evaluate the potential protective effect of *L. kefiri* against the metabolic disorders induced by FRD. *L. kefiri* is a microorganism derived from kefir grains. Previous reports have demonstrated that kefir improves fatty liver syndrome in *ob/ob* mice [47] and metabolic parameters in spontaneously hypertensive rats [48]. Interestingly, in our model the administration of *L. kefiri* completely prevented the alterations caused by FRD intake, which strongly support the beneficial effect of this probiotic. In concordance with our results, other studies have shown that the use of *Lactobacillus* species as probiotics improves the metabolic disorders induced by the FRD [49,50].

There is growing evidence that AT-gut microbiota axis modulates several metabolic processes, including adipokine secretion and lipid metabolism, among others [51]. For this reason, the maintenance of healthier gut microbiota is relevant for the normal function of the AT. It was demonstrated that probiotic administration to high-fat fed mice reduced the infiltration of pro-inflammatory macrophages into AT and also adipocyte size [52]. In line with these findings, our results showed that *L. kefiri* administration to FRD-fed mice decreased the expression of several pro-inflammatory cytokines in EAT, indicating a prevention of the local pro-inflammatory state caused by FRD intake. Previously, it was demonstrated that orally administered *L. kefiri* induces an anti-inflammatory response in the gut of healthy mice [29]. This anti-inflammatory action could lead to the prevention of body weight gain and visceral fat accumulation, as proposed as an explanation for weight modification induced by other lactobacilli [53]. Furthermore, the anti-inflammatory effect was accompanied by a partial attenuation of adipocyte hypertrophy and an improvement of insulin-sensitivity in FRD-Lk adipocytes. It is largely accepted that adipocyte size is directly correlated with LEP secretion [1]. Moreover, LEP induces the secretion of pro-inflammatory cytokines, which in turn have a positive feedback to LEP [43]. It has been reported that periodic administration of probiotic mixture to obese MSG (monosodium glutamate) rats increased adiponectin levels and decreased the leptin concentration in AT and the visceral AT mass [54]. We observed that in vitro FRD-Lk adipocytes secreted an intermediate amount of LEP between CTR and FRD adipocytes, which was in accordance with the partial recovery of adipocyte size in FRD-Lk and the decrease in inflammation. Additionally, expression analysis in EAT from FRD mice that received *L. kefiri* showed lower levels of Ob, but no changes in Adipo mRNA levels compared to EAT from FRD mice. Several studies have demonstrated the beneficial effect of probiotic on lipid metabolism, by regulating the expression of lipid metabolism-related enzymes [55–57]. Specifically, a direct effect on AT lipid metabolism has been observed [58]. Our findings indicate that FRD intake generated an imbalance in the expression of lipogenic/lipolitic enzymes in EAT, that was recovered by *L. kefiri* administration. Overall, our results strongly support that *L. kefiri* treatment has several beneficial effects in AT metabolism and function, suggesting a tight communication between AT and the gut microbiota. However, further studies are needed to elucidate the mechanisms involved.

Different mediators have been proposed as a link between intestinal microbiota and host metabolism. One of these is short-chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, which are generated as a result of bacterial fermentative metabolism. Due to its heterofermentative metabolism, *L. kefiri* produces lactic acid, ethanol and carbon dioxide as main metabolites of sugar fermentation [27]. To our knowledge, there are no scientific reports about SCFA production by *L. kefiri* strains. However, it is very interesting that Iraporda and coworkers recently demonstrated that lactate inhibits the activation of intestinal epithelial cells triggered by different pro-inflammatory stimuli [59,60]. SCFAs have been shown to generate protection against diet-induced obesity [61,62]. Several of their actions are mediated through activation of free fatty acid receptors (FFARs) [63], some of which are abundantly expressed in AT and may be involved in regulating lipid metabolism and glucose homeostasis [64]. Although we did not analyze the presence of lactic acid or SCFAs in fecal samples in our study, we cannot discard the hypothesis that the production of lactate or modifications in SCFA production may contribute to the preventive effect exerted by *L. kefiri* in our model. On the other hand, some trials suggest that another possible mechanism is a lower expression of the tight junction proteins that generates an increased gut permeability to lipopolysaccharides

(LPS). These bacterial ligands can stimulate immune cells, such as those from AT, thus contributing to establish a chronic inflammatory state in obese individuals [41]. In fact, LPS plasma concentrations are increased in obese individuals [65], suggesting its contribution to endotoxemia and AT inflammation development during obesity.

Changes in diversity and number of bacteria in the intestinal microbiota during obesity has been proved, however, no consensus has been reached about the composition of a healthy or unhealthy gut microbiome [66]. In our studies, the PCR-DGGE assay showed that both FRD and Lk administration produced qualitative changes in the microbial community composition, while no changes in diversity were observed. Studies in animal models have shown many controversies related to *Firmicutes* and *Bacteroidetes* abundance, depending on the diet and length of treatment (high fat, high carbohydrate or high fiber diets). Some studies showed that fructose intake produces a decrease in *Bacteroidetes*, while others stated no changes or even an increment [49,67]. Similarly, for *Firmicutes*, an increase or no changes were reported [49,50,67]. In our study, we analyzed if both variables studied (FRD and Lk administration) affected the microbiota composition of treated mice. Firstly, we did not find any changes in *Firmicutes* and *Bacteroidetes* phyla when FRD intake was analyzed. However, we did observe a decrease in fecal *Lactobacillus* spp. in FRD-mice, independently if they received or not *L. kefiri*. This result agrees with those previously shown by Di Luccia *et al.* and Jena *et al.*, who reported a decrease in *Lactobacillus* in high fructose-fed rats [67,68]. When we evaluated the effect of *L. kefiri* administration we observed that it has a significant positive influence in both *Firmicutes* and *Bacteroidetes* phyla, and also in two of the four specific populations studied, *B. fragilis* and *L. murinus*. All these changes evidence an effect per se of the *L. kefiri* strain, independent from the diet, and could be suggesting the establishment of a healthier bacterial community.

As mentioned before, most of the studies evaluating the use of probiotics have been performed in high-fat diet models. However, some works have studied the use of probiotics in high-fructose consumption models and its relationship with changes in microbiota composition. One report showed an increase in both *Firmicutes* and *Bacteroidetes* quantities in small intestine when FRD was co-administered with *L. rhamnosus* GG [49]. In our work, when compared group to group, a significant increase in *Bacteroidetes* was observed in FRD-Lk mice. On the other hand, Zhang et al. reported that FRD supplemented with *L. casei*, increased intestinal *Bacteroides fragilis* (*Bacteroidetes* phylum) and decreased *Clostridium* spp. (*Firmicutes* phylum) quantities, at the same time that it improved the oral glucose tolerance test in FRD-fed rats [50]. In line with these results, FRD-fed mice supplemented with *L. kefiri* showed an increase in *B. fragilis*, but no changes in *Clostridium coccoides* group, accompanied by an improvement of several metabolic alterations caused by FRD intake. Some reports have described beneficial effects of *B. fragilis*, including preventive effects against colitis and intestinal inflammation [69,70], and improvement in plasma levels of triglycerides and glucose [71]. Furthermore, we found that *L. murinus* population was higher in FRD-Lk and CTR-Lk mice. Previously, an increase of *L. murinus* in colon was associated with an improvement in the intestinal immunity [72]. Finally, when we analyzed *Lactobacillus* spp., we found a trend toward increase in CTR-Lk mice, similarly to the significant increase previously reported by our group [29]. It is worth to note that some of these results do not completely agree with Carasi et al. (2015) [29], which could be mostly related to the difference in the length of *L. kefiri* treatment (3 weeks vs. 6 weeks). Overall, these changes in microbiota populations may explain in part the anti-inflammatory and metabolic improvements generated by *L. kefiri* in Lk-treated mice. However, further studies are needed to determine the association between changes in microbiota composition, caused by *L. kefiri* administration, and the metabolic improvements found in our model.

5. Conclusions

In summary, this work showed, as expected, that a fructose-rich diet induced endocrine-metabolic alterations in mice that resemble those found in human MS. These alterations could be partially caused by a dysbiosis induced by FRD; however, more studies about effects of fructose intake on

gut microbiota are needed. In our experimental model, we evidenced that FRD does not alter the *Bacteroidetes* and *Firmicutes* phyla, but decreases *Lactobacillus* spp. Moreover, we demonstrated the beneficial effects of *L. kefiri* as a probiotic, such as changing gut microbiota composition and preventing metabolic alterations and AT dysfunction induced by FRD. In this regard, we previously showed the anti-inflammatory action of *L. kefiri*, accordingly with a novel protective effect against AT inflammation. Finally, we have contributed to reinforce the importance of probiotics as a preventive treatment for metabolic alterations associated with obesity. In particular, the *L. kefiri* strain isolated from a natural food, such as kefir grains, emerges as a potential tool for obesity management.

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Author Contributions: A.G., M.G.Z. and M.d.l.A.S. conceived and designed the experiments. M.G.Z. and S.E.G. performed research. P.C. participated in qPCR assays and analyses. M.A.R. performed the histological analysis. A.G., M.d.l.A.S., S.E.G. and M.G.Z. analyzed all data and wrote the manuscript. All the authors participated in the critical revision and correction of the final manuscript. All authors read and approved the final manuscript.

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

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Review

Targeting Overconsumption of Sugar-Sweetened Beverages vs. Overall Poor Diet Quality for Cardiometabolic Diseases Risk Prevention: Place Your Bets!

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Abstract: Chronic overconsumption of sugar-sweetened beverages (SSBs) is amongst the dietary factors most consistently found to be associated with obesity, type 2 diabetes (T2D) and cardiovascular disease (CVD) risk in large epidemiological studies. Intervention studies have shown that SSB overconsumption increases intra-abdominal obesity and ectopic lipid deposition in the liver, and also exacerbates cardiometabolic risk. Similar to the prevalence of obesity and T2D, national surveys of food consumption have shown that chronic overconsumption of SSBs is skyrocketing in many parts of the world, yet with marked heterogeneity across countries. SSB overconsumption is also particularly worrisome among children and adolescents. Although the relationships between SSB overconsumption and obesity, T2D, and CVD are rather consistent in epidemiological studies, it has also been shown that SSB overconsumption is part of an overall poor dietary pattern and is particularly prevalent among subgroups of the population with low socioeconomic status, thereby questioning the major focus on SSBs to target/prevent cardiometabolic diseases. Public health initiatives aimed specifically at decreasing SSB overconsumption will most likely be successful in influencing SSB consumption per se. However, comprehensive strategies targeting poor dietary patterns and aiming at improving global dietary quality are likely to have much more impact in addressing the unprecedented public health challenges that we are currently facing.

Keywords: sugar-sweetened beverages; diet quality; abdominal obesity; type 2 diabetes; cardiovascular diseases

1. Introduction

The epidemics of obesity, type 2 diabetes (T2D), and cardiovascular diseases (CVD) are affecting most if not all developed countries around the world. While the prevalence of overweight, obesity, and T2D remain high in North America and Western Europe, obesity rates and T2D rates are increasing at a stunning pace in developing countries [1,2]. For instance, a recent study showed that in Mexico City inhabitants aged between 35 and 74 years, the excess mortality associated with previously diagnosed T2D accounted for one third of all deaths between 1998 to 2004 [3]. Added sugar is one of the most consistent dietary features found to be associated with obesity, T2D, and CVD rates in large epidemiological studies [4–7]. In some countries such as the Unites States, sugar-sweetened beverages (SSBs) account for almost half of the added sugar consumed nationally [8]. SSB consumption around

the world has reached unprecedented proportions, and the rise in the prevalence of cardiometabolic risk factors in children such as abdominal obesity and insulin resistance has increased in parallel. SSBs typically include carbonated soft drinks, juice drinks (with added sugars), sports drinks, energy drinks, milkshakes, and iced tea or coffee. A recent modelling study performed by the Global Burden of Diseases Nutrition and Chronic Diseases Expert Group (NutriCoDE) estimated that up to 184,000 deaths per year could be attributed to the chronic overconsumption of SSBs [9]. Similar to the prevalence of obesity and T2D, studies analysing national surveys of food consumption have shown that the chronic overconsumption of SSBs is also skyrocketing in many parts of the world, yet with marked heterogeneity across countries [10].

Although the relationship between SSB overconsumption and obesity and T2D is quite consistent, whether this association reflects a causal link has been debated as some studies have shown that SSB consumption is closely associated with other factors such as poor overall diet quality as well as an unfavourable socioeconomic status. The objective of this review article is to present the latest research on the association between chronic overconsumption of SSBs, diet quality, and cardiometabolic diseases, and to present evidence arguing for and against the potential causal role of SSBs in the aetiology of cardiometabolic diseases.

2. Cardiometabolic Impact of Sugar-Sweetened Beverages Overconsumption

SSBs are the single greatest source of added sugars in most Western countries. SSBs are typically sweetened with high-fructose corn syrup (HFCS) or sucrose. Sucrose, also often referred to as table sugar, is a disaccharide composed of glucose and fructose linked via a glycoside bond. Most sucrose is obtained from sugar cane and sugar beets. Maersk et al. [11] showed in a six-month parallel intervention study of 47 overweight individuals that the consumption of 1 L/day of sucrose-sweetened beverages (cola) significantly increased visceral adipose tissue and hepatic fat accumulation compared to the consumption of 1 L/day of semi-skimmed milk, artificially-sweetened beverages (ASBs), or water. Although not associated with increases in body weight or total fat mass, the consumption of cola was linked with increases in plasma triglyceride and cholesterol levels. Interestingly, daily total energy intake did not appear to differ across subgroups, thereby suggesting that energy included in beverages could have been compensated for by reductions in energy from other sources. HFCS is produced by industrial processing of corn starch. It contains two monosaccharides, free fructose, and glucose in various proportions. Both fructose and glucose have different metabolic fates, an observation that has encouraged many to suggest that fructose may have a unique role in the pathogenesis of cardiometabolic diseases. This hypothesis has been supported by well-designed controlled studies. For instance, Stanhope et al. [12] enrolled 32 men and women who were either overweight or obese to a 10-week randomized clinical trial designed to determine the relative effects of glucose- or fructose-sweetened beverages on visceral adiposity, insulin sensitivity, and lipoprotein-lipid metabolism. During the intervention, study participants were fed an ad libitum diet with 25% of calories originating from glucose- or fructose-sweetened beverages. Although both diets increased body weight, only participants in the fructose group had increased visceral adipose tissue accumulation at the end of the trial. The area under the curve of insulin levels during a 3-h oral glucose tolerance test increased by 27% in the fructose group (significant) and by approximately 14% in the glucose group (nonsignificant). Similarly, 24-h post-prandial triglyceride and fasting apolipoprotein B levels, as well as small, dense low-density lipoprotein (LDL) levels and triglyceride levels in remnant-like particles were all increased in the fructose but not in the glucose group following the intervention. Kinetic studies with isotopic acetate infusions revealed that hepatic fractional de novo lipogenesis increased in the fructose but not in the glucose group, thereby providing evidence that fructose overconsumption could contribute to poor cardiometabolic health. Another investigation using a randomized parallel design by the same group revealed that the consumption of 10%, 17.5%, or 25% of total energy as fructose-sweetened beverages led to dose-dependent increases in plasma lipoprotein-lipid and uric acid levels in as little as two weeks in young adults [13]. Consumption of moderate (40 g/day) and

high (80 g/day) amounts of fructose- and sucrose-sweetened beverages were also found to slightly (but significantly) decrease hepatic insulin sensitivity and increase LDL cholesterol levels (but not triglycerides) in healthy young men compared with similar amounts of glucose-sweetened beverages for three weeks [14]. However, studying the impact of four weeks of fructose- and glucose-sweetened beverages under hypercaloric diets (150 g/day), Silbernagel et al. [15] found no group differences with regards to visceral or hepatic fat accumulation, plasma insulin, or cholesterol levels between both study groups. Only triglyceride levels appeared to have increased following the fructose-based diet. Overall, most but not all of the above studies suggest that consumption of SSBs, which corresponds to or is slightly higher than a high SSB consumption in the general population, may be associated with visceral adiposity/ectopic lipid deposition, insulin resistance, and an impaired lipoprotein-lipid profile. However, it is stressed that most of these studies were performed over relatively short periods, by randomized trials that had limited sample sizes and have not all adequately accounted for concurrent changes in total calories intake.

3. Sugar-Sweetened Beverages Overconsumption and Cardiometabolic Diseases Risk

About a dozen large prospective epidemiological studies have documented the association between SSB consumption and the risk of cardiometabolic diseases such as obesity, metabolic syndrome, T2D, and CVD. This literature has recently been extensively reviewed by Malik and Hu [16]. In 2013, these investigators performed a meta-analysis that included 25,745 children and adolescents from 15 prospective studies and 174,252 adults from in seven prospective studies [17]. The results of this analysis suggest that a one serving per day increase in SSB is associated with a 0.06 unit increase in body mass index (BMI) per year in children and adolescent and with a 0.12 to 0.22 kg yearly weight gain in adults. Although this study clearly shows that a high consumption of SSBs might promote weight gain in children and adults, the impact of a moderate consumption of SSBs (between one serving per day and one serving per week) on long-term body weight changes could not be modelled. In addition to this report on weight gain, the same group also published evidence that SSBs overconsumption is linked with the onset of the metabolic syndrome (a constellation of CVD and T2D risk factors associated with abdominal obesity and insulin resistance) and T2D [7]. This meta-analysis included 310,819 participants and 15,043 cases of T2D from nine cohorts. Compared to individuals in the lowest SSB consumption quintile, those in the top quintile had a multivariable adjusted relative risk of developing T2D of 1.26 (95% confidence interval, 1.12–1.41). The analysis on the incidence of metabolic syndrome included 19,431 participants and 5803 incident cases of metabolic syndrome from three cohorts. Compared to individuals in the lowest SSB consumption quintile, those in the top quintile had a multivariable adjusted relative risk of developing the metabolic syndrome of 1.20 (95% confidence interval, 1.02–1.42). Shortly after, investigators of the PREDIMED trial reported similar findings [18]. In this cohort, overconsumption of SSBs ($\geq$5 servings per week vs. 0 servings per week) was positively associated with the incidence of the metabolic syndrome after multivariable adjustment (hazard ratio = 1.43 (95% confidence interval, 1.00–2.15)) while a moderate consumption (1–5 servings per week) was not (hazard ratio = 0.91 (95% confidence interval, 0.74–1.12)). Interestingly, the prevalence of individuals reporting consuming $\geq$5 SSB servings per week in this Mediterranean population ranged between 5% and 10%. Individuals reporting consuming $\geq$5 SSB servings per week were also less likely to adhere to the Mediterranean diet and consume less fruit, while being more likely to consume baked products, alcohol, and a higher total daily energy compared to other study participants. Another recent meta-analysis by Imamura et al. [4] revealed that higher consumption of SSBs was associated with a higher T2D risk (18% increase in risk per serving per day), independently of adiposity. This analysis, which was based on 38,253 cases of T2D, also revealed that ASBs as well as fruit juice consumption could be linked with T2D incidence, although the risk estimates were not as high compared to SSBs and the associations were likely to involve bias according to the authors. In an effort to further support the link between SSB consumption and non-alcoholic fatty liver disease (NAFLD), Wijarnpreecha et al. performed a meta-analysis of seven (mostly cross-sectional) studies

and reported that individuals consuming SSBs were at increased risk of NAFLD compared to those not reporting consuming SSBs (hazard ratio = 1.53 (95% confidence interval, 1.34–1.75)) [19].

Similar measures of associations were recently reported for CVD incidence in the meta-analysis of Narain et al. [20]. This study included results from 308,420 individuals from seven prospective cohort studies. Compared to individuals with a low SSB consumption (usually in lowest quartiles or quintiles), those who reported the highest SSB consumption (usually in the top quartiles or quintiles) had a relative risk of 1.10 (95% confidence interval, 1.02–1.18) for total cardiovascular events. Interestingly, the risk associated with an elevated consumption of SSB was higher for myocardial infarction (hazard ratio = 1.19 (95% confidence interval, 1.09–1.31)) compared to stroke (hazard ratio = 1.10 (95% confidence interval, 0.97–1.25)), other vascular events (hazard ratio = 1.09 (95% confidence interval, 0.82–1.45)), or mortality (hazard ratio = 1.03 (95% confidence interval, 0.91–1.18)). Other meta-analyses also reported small but significant associations between SSB consumption and the risk of hypertension [21] as well as the risk of chronic kidney disease [22].

The above studies have established a clear association between SSB consumption and a broad range of cardiometabolic diseases. However, it is worth highlighting that these studies share the well-known limitations of observational studies. For instance, although some consideration is given to potential confounding factors (age, smoking, socioeconomic status, cardiometabolic risk factors, total energy intake, alcohol consumption, intake of other foods, etc.) in the regression models, the issue of residual confounding cannot be fully addressed, which could lead to an inaccurate estimation of the reported measures of associations. Many confounders are often imprecisely measured in large datasets and other confounders are often not even measured or considered. A proper assessment of physical activity/sedentarity is also lacking in most studies. It is also worth highlighting that few "dose-response" associations with the risk of cardiometabolic diseases have been reported. For instance, the results of the majority of observational studies suggest that individuals in the top quartiles or quintiles of SSB consumption are at elevated risk of obesity, T2D, or CVD, while individuals in other groups may not have been at increased risk, thereby highlighting that SSB overconsumption may be potentially harmful while a moderate consumption (less than one per day but more than one per week) may not be. Additionally, although results from the above studies consistently suggest that individuals in the highest SSB consumption categories may be at risk, with elevations in risk ranging from 10% to 30%, this level of risk could be qualified as at best modest.

4. Is It the Sugar-Sweetened Beverages per Se or Are They Partners in Crime?

Few studies have directly compared the impact of SSB consumption to other foods regarding the risk of developing cardiometabolic diseases over long periods of time. One of these studies revealed that each daily SSB serving was associated with a one-pound increase in body weight over a four-year period after adjusting for age, baseline BMI, and other potential confounders including many dietary items in a pooled analysis from the Nurses' Health Study (I and II) and the Health Professionals Follow-up Study [23]. This study also revealed that the dietary items that were associated with the highest weigh gain over the same period were French fries (3.35 lb), potato chips (1.69 lb), potatoes (1.28 lb), as well as unprocessed and processed red meats (0.95 and 0.93 lbs, respectively). Interestingly, food items found to be negatively associated with weight gain included yoghurt (−0.82 lb), nuts (−0.57 lb), fruits (−0.49 lb), whole grains (−0.37 lb), and vegetables (−0.22 lb). It is interesting to note that many of the foods found to be negatively associated with weight gain contained various amounts of sugar, thereby highlighting that sugar consumption per se may be negatively or positively associated with weight gain, depending on its food source. In a recent modelling analysis based on the National Health and Nutrition Examination Surveys and several of the above-stated meta-analyses, Micha et al. [24] reported that the over- or the under-consumption of 10 dietary factors could explain up to 45% of cardiometabolic deaths (from coronary heart disease, stroke, or T2D). Dietary factors positively linked with cardiometabolic deaths included sodium, processed and unprocessed meats,

and SSBs, while factors negatively associated with cardiometabolic deaths include polyunsaturated fats, nuts and seeds, whole grains, fruits, vegetables, and seafood omega-3 fats.

SSBs, fried foods, processed and unprocessed red meats, refined grains, snacks, and desserts are all part of the so-called Western diet. People who adhere to this pattern also tend to eat more calories per day compared to those who adhere to a prudent dietary pattern, a pattern associated with a higher overall dietary quality that is recommended in most dietary guidelines around the world. Interestingly, individuals who report adhering to a Western dietary pattern also tend to report eating out of home more often. This dietary pattern is also negatively correlated with socioeconomic status and is increasingly observed in developing countries [25], which is particularly worrisome given its strong association with the long-term risk of T2D and CVD [26,27].

One of the reasons why many public health advocates rapidly point the finger at SSBs to identify them as the main (and in some cases the only) driver of cardiometabolic disease is based on the fact that SSBs, unlike other constituents of the Western dietary pattern, provide a significant amount of extra or "unnecessary" calories to the human body as these calories do not contribute to satiety and may even promote overeating [28]. Those calories consumed on a regular basis do contribute to a positive energy balance and long-term weight gain and cardiometabolic risk. Whether the weight gain associated with SSB consumption is the result of more added sugars to one's diet or whether simply the extra calories from SSBs contribute the weight gain is still under debate, and it is beyond our objective to address this issue as others have recently thoroughly addressed this timely topic [29–31].

In order to determine whether SSB consumption is an actionable risk factor that should be targeted for the prevention and/or management of cardiometabolic diseases, the potential confounding factors mediating the relationship between SSBs and the risk of cardiometabolic diseases need to be considered. This is best illustrated by the Kangbuk Samsung Health Study, which has recently documented the relationship between SSB consumption and coronary atherosclerosis measured by computed tomography in a cross-sectional study that included 22,210 men and women with food frequency questionnaire (FFQ) data available [32]. In this study, compared to individuals who reported never consuming sugar-sweetened carbonated drinks, those who reported consuming $\geq$5 per week had an odds ratio for the presence of coronary artery calcium of 1.31 (95% confidence interval, 1.06–1.62). Also in this study, those who reported drinking between 3 and 5 sugar-sweetened carbonated drinks per week did not have a higher risk for the presence of coronary artery calcium (odds ratio = 0.88 (95% confidence interval, 0.72–1.07)), a finding that further highlights the notion that moderate SSB consumption may not cause atherosclerotic CVD. Interestingly, compared to individuals who reported never consuming sugar-sweetened carbonated drinks, those who reported consuming $\geq$5 per week ate approximately 29% more calories per day, 52% more red and processed meat, and 60% more sugary foods. These individuals were also twice as likely to be smokers. Adjusting for some of these risk factors attenuated the association between SSB consumption and the presence of coronary artery calcium. The same is true for the variables that may predict SSB consumption.

In this regard, it has been known for decades that most dietary habits are formed during early childhood, and the same holds true for SSB consumption. A recent systematic review of the literature published aimed at identifying the determinants of SSB consumption in children under 7 years of age. Reviewed articles included reports from intervention, prospective, and cross-sectional studies. Many factors associated with elevated SSB consumption in children were identified such as child's preference for SSBs, child television viewing/screen time, child snack consumption, parents' lower socioeconomic status, parents' younger age, parents' SSB consumption, formula milk feeding, early introduction of solids, using food as rewards, parental-perceived barriers, attending out-of-home care, and living near a fast-food/convenience store. Additional factors were associated with lower SSB consumption in children, such as parental positive modelling, parents being married/cohabiting, school nutrition policy, and living near a supermarket. Results of this study suggest that drivers of SSB consumption are multifactorial. It also suggests that there are strong interrelationships among these determinants and that targeting only one of them in isolation without the others might not

be effective in reducing SSB consumption in children or at the population level, although this still needs to be demonstrated. In this regard, a recent study confirmed a significant decline in added sugars consumption and availability in Australia between 1995 and 2011–2012 using four independent datasets without significant declines in obesity rates. Figure 1 presents a schematic representation of the correlates of SSB consumption, confounding factors that mediate the relationship between SSB consumption and the risk of cardiometabolic diseases, as well as the factors that predict SSB consumption, overall highlighting the notion that a comprehensive set of barriers and facilitators will need to be considered if we aim to reduce SSB consumption at the population level.

Figure 1. Schematic representation of factors associated with sugar-sweetened beverage (SSB) consumption.

5. Disparities in Sugar-Sweetened Beverage Overconsumption around the World

Almost all the studies presented and discussed above were performed in homogeneous economically developed countries. While the evidence suggests that the deleterious impact of chronic overconsumption of SSB is independent of ethnic background, population-wide levels of SSB consumption are extremely heterogeneous across countries and could explain why some countries may be more likely than other to witness changes in cardiometabolic diseases incidence compared to others over the next few years. In this regard, the NutriCoDE study group [33] recently presented data on SSB consumption across different age ranges and sex from 51 countries representing 63% of the world's population based on national or sub-national diet surveys for the year 2010. Results of this study suggest that regions of the world with the highest consumption of SSBs included Latin America, the Caribbean, and North America, while the lowest consumption is recorded in East Asia. In some parts of Latin America, the average SSB consumption exceeded 3 servings/day. Popkin and Hawkes [34] recently investigated the 2009–2014 changes in SSB sales in different regions of the world using data from the Euromonitor Passport International Database. Their investigation revealed that

SSB sales increased in most low-income and middle-income countries, with Chile being the country with the most important rise in SSB sales during this period. Interestingly, they showed that SSB sales decreased in many high-income regions such as North America, Australasia, and Western Europe, although they remain on average very high compared to other countries. Considering the uncertainties with regard to the potential independent impact of SSBs on obesity levels, this observation suggests that consumers are probably more aware of the detrimental effects of chronic overconsumption of SSBs (despite them being heavily marketed compared to healthier alternatives). Under the hypothesis that SSB consumption is a strong and independent determinant of obesity, such a massive reduction in SSB sales observed at nation-wide levels should be associated with decreases in overweight/obesity. However, the prevalence of overweight/obesity has not decreased over the same period; rather it has increased significantly.

To further illustrate this point, we have also turned to the Euromonitor International Passport Database and obtained SSB sales (soft drinks, juice drinks, energy drinks, and sports drinks) between 2002 and 2014 together with the prevalence of overweight/obesity in selected countries across most continents (from the World Health Organisation). Our results, presented in Figure 2, show that among countries of different continents with heterogeneous populations and socioeconomic statuses, the country-level SSB sales are very heterogeneous. In Western populations such as in the Unites States and Australia, SSB sales have significantly decreased, while they have increased significantly in other countries such as the United Kingdom, Chile, Saudi Arabia, South Africa, Thailand, and Mexico. Parallel to these changes, the percentage of individuals who were considered to be overweight or obese (BMI $\geq$ 25 kg/m^2) increased everywhere, regardless of country-level SSB sales. This "pragmatic" observation also highlights the point that targeting SSBs in isolation without other efforts to improve overall nutritional quality may not lead to lasting changes in the prevalence of overweight/obesity.

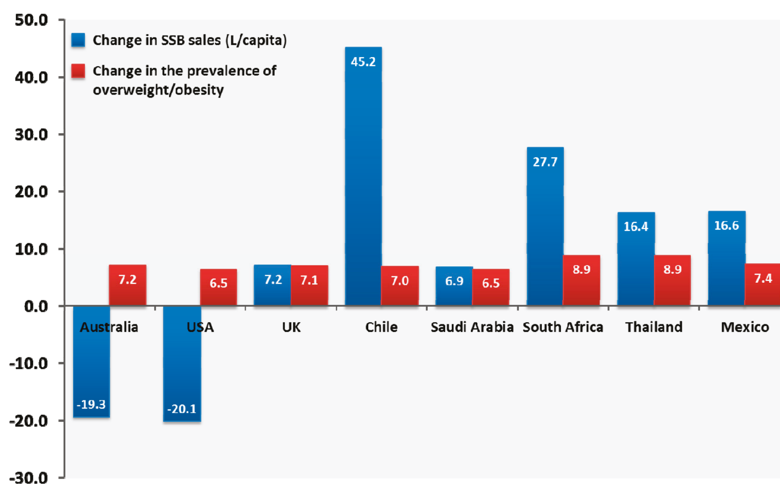

Figure 2. 2002–2014 changes in sugar-sweetened beverage sales presented in litres per capita, and 2002–2014 changes in the percentage of the population who is either overweight or obese (body mass index $\geq$ 25 kg/m^2) in selected countries from various regions of the world. Sugar-sweetened beverages were defined as the sum of on trade (bars, cafés, restaurants, etc.) and off trade (grocery stores, independent retailers, etc.) sales in any sugar-sweetened sodas (regular cola carbonates, lemonade/lime carbonates, ginger ale, tonic water, and orange carbonates), juice drinks (up to 24% juice), and nectars (25–99% juice), as well as sports drinks and energy drinks. Low-calorie cola carbonates and 100% fruit juices were not included. Data on body mass index changes were obtained from the World Health Organisation website.

As previously stated, SSB consumption is strongly associated with many confounding factors such as a Western type diet, ultra-processed food consumption, overall poor diet quality, smoking, less exercise, etc., which has led many to suggest that SSBs may also be a marker of poor lifestyle habits rather that an actionable risk factor for cardiometabolic diseases. Although cross-sectional studies have suggested that SSB overconsumption is correlated with poor diet quality [35], one argument against SSBs being a marker of nutritional quality is that national SSB sales/consumption appears to be decreasing in the absence of meaningful changes in nutritional quality [36]. A recent study also showed that a six-month intervention aiming at decreasing SSB consumption in a sample of adults with a high prevalence of obesity was not linked with improvements in other components of the diet (except vegetables consumption, which increased significantly from baseline) [37].

In developing countries, however, the rise in SSB sales/consumption appears to increase in parallel to the rise in ultra-processed food consumption [38,39]. Altogether, these observations suggest that the Western population seems to be increasingly aware of the detrimental impact of SSB overconsumption, and the consumption of SSBs appears to be increasingly "denormalised." As countries in North America and Western Europe where SSB consumption appeared to be decreasing only accounts for about 10–20% of the world population at best, these gains will eventually need to be applied to the rest of the world and, more importantly, be coupled with improvements in overall dietary quality to address the epidemics of cardiometabolic disease.

6. Impact of Decreasing Sugar-Sweetened Beverage Intake

Although decreases in SSB consumption may not correlate with reductions in obesity rates at the national level, several studies have shown in intervention trials that decreasing SSB consumption may be associated with cardiometabolic benefits. In 2012, de Ruyter et al. [40] showed that masked replacement of SSBs with ASBs reduced weight gain in healthy Dutch children. This school-based intervention trial included 641 normal weight children aged between 5 and 12 years of age, randomized to receive 250 mL of SSBs (104 calories) or 250 mL of ASBs (0 calories) per day for 18 months. During the trial, children randomized to SSBs gained 7.37 kg on average, while children randomized to ASBs gained 6.35 kg on average. In the same issue of the New England Journal of Medicine, Ebbeling et al. [41] reported the results of another SSB reduction randomized clinical trial conducted in adolescents. This trial included 224 adolescents who were either overweight or obese who reported consuming at least one serving of SSBs or 100% fruit juice per day, and was designed to last two years. During the first year, adolescents randomized to the experimental group were advised to stop drinking SSBs as part of a multifactorial intervention during which water and ASBs were provided and were followed during the second year without intervention. The primary outcome of the study was change in BMI at two years. The intervention was very successful at reducing SSB consumption. At one year, adolescents included in the control group gained weight while those included in the intervention did not. However, changes in BMI at two years were comparable between the intervention and control groups. This suggests that intervention targeting SSB consumption in adolescents may not be successful if they are not sustained over long periods of time. Another study conducted in 1140 Brazilian fourth graders revealed that an education program aimed at discouraging children to drink SSBs during a full school year led to significant changes in SSB consumption (−56 mL per day on average) while having no effect on BMI, even in children who were characterized by excess body weight at baseline [42].

Altogether, these findings suggest that interventions aimed at reducing SSB intake on body weight have yielded conflicting and inconsistent results. However, given the somewhat moderate association between BMI and the risk of T2D and CVD as well as the results of recent studies that have shown that a more refined assessment of body composition or body fat distribution such as visceral fat or hepatic fat accumulation [43,44], measuring or judging the efficacy of SSB reductions by their effect on total body weight could be misleading. In this regard, several cross-sectional studies have shown that individuals who report consuming SSBs on a daily basis carry more visceral and liver fat accumulation, regardless

of body weight [45–48]. In the Framingham Heart Study, determining the associations between SSB consumption on six-year changes in visceral adipose tissue accumulation, Ma et al. [49] reported that individuals who reported consuming SSBs on a daily basis gained approximately 30% more visceral fat compared to individuals who did not consume SSBs, even after adjustment for potential confounders including changes in body weight. Further, Campos et al. [50] recently showed in a randomized clinical trial that included 27 men with a BMI greater than 25 kg/m^2 with a high consumption of SSBs that substituting SSBs with ASBs was associated with a 74% reduction of intra-hepatic lipids (measured by magnetic resonance spectroscopy) in just 12 weeks. At the end of the trial, participants randomized to continue their habitual consumption of SSBs were consuming more than 600 kCal/day on average compared to participants randomized to ASBs. Although an important effect on liver fat accumulation was noted, body weight, visceral adiposity, insulin sensitivity, and (unexpectedly) post-prandial triglyceride levels did not improve in the 12 weeks following this substitution.

Results of the above studies suggest that assessing key components of body fat distribution such as visceral and hepatic fat may prove to be crucial to properly evaluate the effect of SSB reduction on cardiometabolic health. Additionally, given the notion that a poor overall dietary quality is likely to contribute more to cardiometabolic disease risk than individual dietary factors such as SSBs [24], it is unclear how targeting only SSBs without improving overall dietary quality will prove to be beneficial. In this regard, studies are urgently needed to determine whether targeting SSBs, with or without parallel improvements in overall dietary quality, could influence body fat distribution and cardiometabolic health in order to determine whether targeting SSB intake in isolation or in conjunction with other changes aiming at improving dietary quality will result in lasting changes in cardiometabolic health in children, adolescents, and young adults (who are the greatest consumers of SSBs) [33].

7. Conclusions

Obesity and T2D rates are increasing in almost all jurisdictions around the world and will likely cause prejudice to improvements in cardiovascular mortality rates that we have seen decline since the 1970s. Obesity and T2D are complex diseases that are explained by a variety of factors, some related to food consumption (high energy intake, poor dietary quality/ultra-processed foods consumption, food marketing, food availability, culture, etc.) and some less related to diet such as genetic and epigenetic factors, neurobiological factors, adipose tissue function, gut microbiota, lack of physical activity/exercise, screen and sitting time, the built environment, lack of sleep, air pollution, and socioeconomic status, etc. Such a complex interplay of factors make it unlikely that putting the focus on one macronutrient (sugar) or one food item (SSBs) will solve the obesity and T2D epidemics. Nevertheless, the chronic overconsumption of SSBs is associated with a broad range of cardiometabolic diseases such as abdominal obesity, hepatic fat accumulation, metabolic syndrome, T2D, and CVD. Although individuals who consume small quantities or no SSBs at all have a better prognosis than individuals who consume SSBs on a daily basis, it is unsure whether SSBs alone are a driver of poor cardiometabolic health or merely a marker of an overall poor dietary quality or even of a lower socioeconomic status associated with high risk lifestyle habits. Therefore, interventions aimed at targeting overall dietary quality and the determinants of SSB consumption are more likely to lead to lasting changes in SSB consumption and to, overall, more substantial improvements in cardiometabolic health. It is encouraging to note that both SSB consumption and the vast majority of the determinants of SSB consumption are modifiable. However, in order to modify them, unprecedented interventions targeting children directly, their parents, as well as their school, workplace, and built environment will likely be required if we want to be serious in our efforts to reduce SSB consumption, improve dietary quality, and ultimately reduce the risk of cardiometabolic diseases at the population level.

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Review

Effects of Natural Products on Fructose-Induced Nonalcoholic Fatty Liver Disease (NAFLD)

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Abstract: As a sugar additive, fructose is widely used in processed foods and beverages. Excessive fructose consumption can cause hepatic steatosis and dyslipidemia, leading to the development of metabolic syndrome. Recent research revealed that fructose-induced nonalcoholic fatty liver disease (NAFLD) is related to several pathological processes, including: (1) augmenting lipogenesis; (2) leading to mitochondrial dysfunction; (3) stimulating the activation of inflammatory pathways; and (4) causing insulin resistance. Cellular signaling research indicated that partial factors play significant roles in fructose-induced NAFLD, involving liver X receptor (LXR)α, sterol regulatory element binding protein (SREBP)-1/1c, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD), peroxisome proliferator–activated receptor α (PPARα), leptin nuclear factor-erythroid 2-related factor 2 (Nrf2), nuclear factor kappa B (NF-κB), tumor necrosis factor α (TNF-α), c-Jun amino terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K) and adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK). Until now, a series of natural products have been reported as regulators of NAFLD in vivo and in vitro. This paper reviews the natural products (e.g., curcumin, resveratrol, and (−)-epicatechin) and their mechanisms of ameliorating fructose-induced NAFLD over the past years. Although, as lead compounds, natural products usually have fewer activities compared with synthesized compounds, it will shed light on studies aiming to discover new drugs for NAFLD.

Keywords: fructose-induced NAFLD; lipogenesis; natural products; mitochondrial dysfunction; inflammatory pathways; insulin resistance

1. Introduction

Fructose—also known as fruit sugar—is a ketonic monosaccharide present in many plants, such as sugar cane, sugar beets, and corn. It has been appreciated for many years that fructose is applied as a sugar additive, typically in high fructose corn syrup, the main ingredient of soft drinks, pastries, desserts, and other daily processed foods. It is postulated that large quantities of added sugars entering the daily diet will enhance the possibility of suffering from nonalcoholic fatty liver disease (NAFLD) [1,2]. Randomized clinical trials revealed that a reduction of sugar-sweetened beverage intake for 6 to 24 months significantly reduced individual body weight gain [3]. Exploratory observational pilot studies showed that over-consumption of fructose in NAFLD patients may increase risk of liver

steatosis [4], up-regulate circulating adropin concentrations [5] and lipocalin 2 level [6,7], increase hepatic fibrosis [8], and finally lead to cirrhosis and liver failure.

Many of clinical trials and ecological studies have assessed the close relationship between excessive consumption of fructose and NAFLD. It has been reviewed that overeating fructose may be the "caries" at the epidemic's root [9]. The majority of the metabolic disorder of fructose is related to its rapid utilization through the liver, leading to increasing lipogenesis and very low density lipoprotein (VLDL) secretion, which finally causes various liver dyslipidemia [10]. Fructose enters hepatocytes through a transporter, especially glucose transporter 2 (Glut2), Glut5, Glut8 [11], or possibly Glut9 [12], where it is preferentially metabolized by fructokinase to generate fructose-1-phophate, then serves as a relatively unregulated source providing carbon atoms for both the glycerol and the acyl portions of triglycerides [13]. Consequently, fructose is a highly efficient inducer of de novo lipogenesis, which has been shown to reduce hepatic insulin sensitivity and increase the formation of VLDL [14]. The major clinical diseases based on hepatic dyslipidemia include obesity, NAFLD, and multiple metabolic syndromes, among which the prevalence of NAFLD has become a global problem in recent decades. It is reported that about 30% of the population in Western countries has been affected with NAFLD, and up to 15% among the overseas Chinese community [15]. Patients with NAFLD tend to suffer from non-alcoholic steatohepatitis, hepatic fibrosis, cirrhosis, hepatoma, and metabolic syndrome, meaning that it is a leading health risk in this population [16,17]. The incidence of metabolic syndrome distributes widely around the world, and the number of people with this syndrome has risen in epidemic proportions during the last 50 years [18]. It is necessary to certify clinical recommendations that fructose intake be controlled by limiting the consumption of sugar (especially high fructose containing)-sweetened foods or drinks. With little effective medicines discovered, there are a certain number of natural products reported to show bioactivity in depressing fructose-induced NAFLD. This paper reviewed the bioactive natural products on NAFLD recently reported in the literature, which can serve as a lead for new drugs research and diary supplement development.

2. Mechanisms of Fructose Action in NAFLD

Fructose is principally metabolized by fructokinase to generate fructose-1-phophate, irregularly providing glycerol phosphate and acyl coenzyme A, resulting in triglyceride formation that is both secreted and stored in hepatocytes [12,13]. Excessive fructose intakes adversely impact hepatic lipid metabolism and insulin sensitivity. Over-loaded fructose increases hepatic metabolic burden, stimulating the overproduction of acetyl-CoA in mitochondria [10]. Through tricarboxylate transport system, acetyl-CoA enters the cytoplasm to be used for fatty acid and cholesterol synthesis by regulation of liver X receptor (LXR)α, sterol regulatory element binding protein (SREBP)-1/1c, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD).

Consequently, increasing lipogenesis in the liver leads to the intracellular accumulation of malonyl-CoA, which represents a balance between synthesis from acetyl-CoA by ACC and utilization in fatty acid synthesis by FAS, as well as degradation to acetyl-CoA via the action of malonyl-CoA decarboxylase. Finally, an excess of malonyl-CoA leads to abnormal production of leptin [19], inhibits hepatic lipid β-oxidation, and increases the formation of reactive oxygen species (ROS), causing mitochondrial dysfunction with down-regulation of peroxisome proliferator-activated receptor α (PPARα)—a major transcription factor involved in the regulation of fatty acid oxidation. Due to the inhibition of nuclear factor-erythroid 2-related factor 2 (Nrf2), antioxidant capability is attenuated, and oxidative stress is thus implacable. Furthermore, ROS triggers inflammatory pathways involving nuclear factor kappa B (NF-κB), tumor necrosis factor α (TNF-α), and c-Jun amino terminal kinase (JNK), leading to hepatic inflammation via the insulin receptor signaling pathway, and further decreases insulin sensitivity, resulting in hepatic insulin resistance [20]. High de novo lipogenesis and oxidative stress are also connected with a deficiency of insulin sensitivity through phosphoinositide 3-kinase (PI3K) and adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) pathways [21–23] (Figure 1).

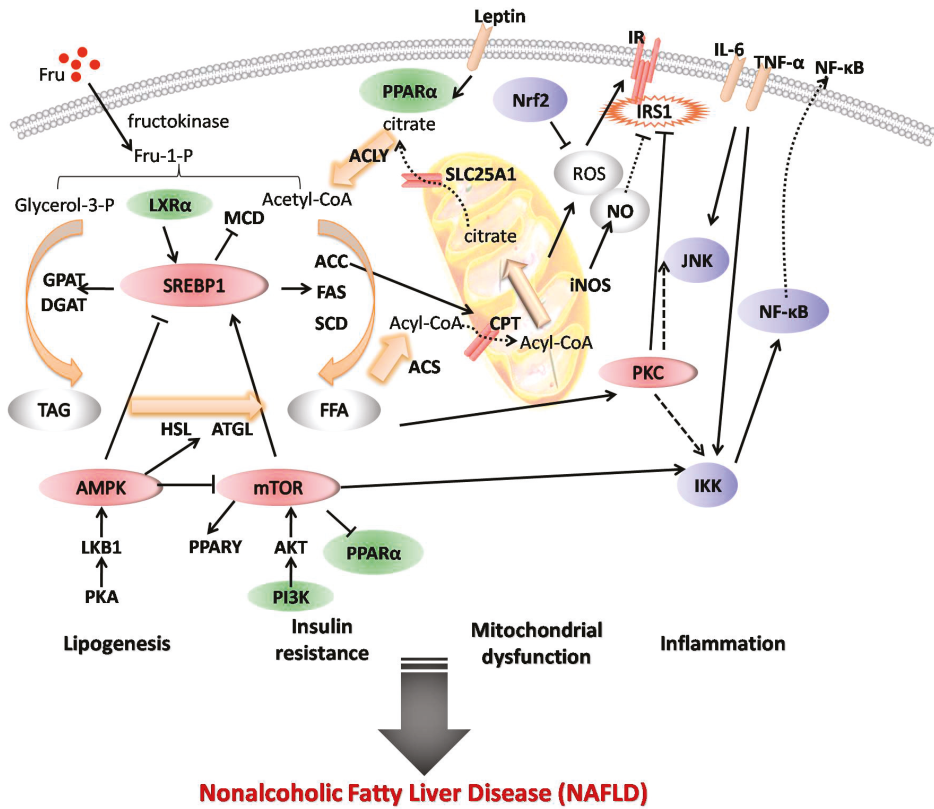

Figure 1. Schematic representation of underlying mechanisms of fructose-induced NAFLD. Fructose may stimulate NAFLD through different pathological processes, including: (1) augmenting lipogenesis through up-regulating liver X receptor (LXR)α and sterol regulatory element binding protein (SREBP)-1c; (2) leading to mitochondrial dysfunction by depressing peroxisome proliferator-activated receptor α (PPARα) and nuclear factor-erythroid 2-related factor 2 (Nrf2); (3) stimulating the activation of inflammatory pathways; and (4) causing insulin resistance. ACC: acetyl-CoA carboxylase; ACLY: ATP-citrate lyase; AKT: protein kinase B; AMPK: adenosine 5′-monophosphate (AMP)-activated protein kinase; ATGL: adipose triglyceride lipase; CPT: carnitine palmitoyl transferase; DGAT: diacylglycerol acyltransferase; FAS: fatty acid synthase; Fru: fructose; FFA: free fatty acid; GPAT: glycerol-3-phosphate acyltransferase; HSL: hormone-sensitive lipase; IKK: inhibitor of nuclear factor κB (IκB) kinase; IL-6: interleukin 6; iNOS: inducible nitric oxide synthase; IRS1: insulin receptor substrate-1; JNK: c-Jun amino terminal kinase; LKB1: Liver kinase B1; MCD: malonyl-CoA decarboxylase; mTOR: mammalian target of rapamycin NF-κB: nuclear factor kappa B; NO: nitric oxide; PI3K: phosphoinositide 3-kinase; PKA/C: Protein kinase A/C; ROS: reactive oxygen species; SCD: stearoyl-CoA desaturase; TAG: triacylglycerol; TNF-α: tumor necrosis factor α.

Solid lines with arrowheads indicate known signaling events and direct stimulatory modification between lipogenesis, mitochondrial dysfunction, inflammation, and insulin resistance. Solid lines with short beelines represent direct inhibitory modification. Dashed lines with arrowheads denote tentative or indirect stimulatory modification. Dashed lines with short beelines mean tentative or indirect inhibitory modification.

3. Effects of Natural Products on Fructose-Induced NAFLD

Many natural products are reported to show regulation effects on fructose-induced NAFLD. Several promising drugs based on studies in rodents have been reviewed against fructose-induced fatty liver and endotoxins influx from the intestine [24]. According to the pathogenesis of fructose-induced NAFLD, the relevant natural products can be classified by their different functions, including regulation of lipogenesis, repair of mitochondrial dysfunction, inhibition of inflammatory pathways, and improvement of insulin resistance.

3.1. Regulation of Lipogenesis

Over-consumption of fructose causes an excess of acetyl-CoA in the mitochondria entering the cytoplasm to be used for fatty acid, leading to over-lipogenesis in the liver. Many natural products show a regulation effect on fructose-induced lipogenesis.

Symplocos cochinchinensis—a popular Indian herbal medicine belonging to the family Symplocaceae—has been utilized for the treatment of diabetes. Ethanol extract of *S. cochinchinensis* was reported to down-regulate lipogenesis and enhance insulin sensitivity of a rat model fed with a high fructose and saturated fat diet. Hepatic gene expression and protein profiles were analyzed after administration of the ethanol extract. The results indicated that *S. cochinchinensis* ethanol extract decreased the expression of SCD-1, SREBP-1c, and FAS to modulate lipid accumulation and attenuate hepatic insulin resistance [25]. Another traditional herbal medicine for obesity and diabetes—the root of *Salacia oblonga*, belonging to the family Celastraceae—exhibited a regulation effect on fructose-induced fatty liver in rats. The aqueous-ethanolic extract of *S. oblonga* root diminished lipid steatosis by reducing excess triglyceride accumulation and preventing the increased hepatocellular vacuolization. The fructose-stimulated overexpression of relevant genes was suppressed, such as SREBP-1c, FAS, ACC-1 and SCD-1 mRNA, suggesting that the modulation of the extract is mediated by hepatic SREBP-1c [26]. In addition, the lipid-lowering effect of green tea extract is also partly mediated by its inhibition of liver SREBP-1c and the responsive genes involving FAS and SCD-1 in fructose-fed rats [27].

Besides, it has been pointed out that compounds isolated from herbal medicines show certain bioactivity in controlling hepatic lipogenesis. Silymarin—a flavonolignan isolated from *Silybum marianum*—has emerged as a potential hepatoprotective agent, and was also effective in reducing de novo hepatic lipogenesis. Silymarin treatment significantly depressed the upregulation of SREBP-1c, LXRβ, and FAS genes in the liver of rat model induced by high fructose diet, associated with recovery in insulin sensitivity [28]. Curcumin—a phenolic compound isolated from *Curcuma longa*—has obvious antioxidant and anti-inflammatory properties, which can also prevent high-fructose-induced hyperlipidemia and hepatic steatosis. Administration of curcumin not only obviously lowered triglyceride content and decreased the hepatic protein expression of LXRα and SREBP-1c, but also suppressed the expression of lipogenic enzymes, including ATP-citrate lyase, ACC, and FAS in rats treated with a high-fructose diet [29]. Oleanolic acid—a pentacyclic triterpenoid compound widely distributed in various plants—is clinically applied for hepatoprotective effect in China. This compound showed an inhibitory effect on triglyceride accumulation in fructose-induced rats. The modulation of oleanolic acid was mediated by down-regulating the mRNA expression of SREBP-1c and its nuclear protein expression, which is responsible for de novo lipid synthesis [30]. The anti-steatotic effect of several natural compounds may occur independently of the hepatic signals associated with de novo fatty acid synthesis. For example, mangiferin—a xanthone glucoside derived from *Mangifera indica* and root of *Anemarrhena asphodeloides*—diminished fatty liver in rats treated with fructose by inhibiting hepatic diacylglycerol acyltransferase-2 that catalyzes the final step in triglyceride biosynthesis to reduce the accumulation of triglyceride, instead of SREBP-1c, FAS, ACC-1, and SCD-1 [31]. Genistein [32] and β-conglycinin [33] are also reported to have a regulation effect on fructose-induced hepatic lipogenesis.

3.2. Repair of Mitochondrial Dysfunction

An excessive intake of fructose gradually leads to an excess of malonyl-CoA, inhibiting hepatic lipid β-oxidation. The oxidative stress caused by lack of antioxidant capability can be diminished by some natural products targeting leptin, PPARα, and Nrf2.

Aqueous seed extract of *Hunteria umbellata* (belonging to the family Apocynaceae) significantly ameliorated the alterations of blood glucose, insulin, leptin, cholesterol, and triglycerides in high-fructose diet-induced metabolic syndrome rats. Moreover, the extract increased the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose 6-phosphate dehydrogenase, and glutathione levels [34]. Polyphenols-enriched extracts from hawthorn fruit peels and fleshes (belonging to Rosaceae) were found to mitigate liver oxidative stress induced by high-fructose diet in mice. Administration of the extracts elevated antioxidant enzyme activities and up-regulated PPARα expression, while down regulating Nrf-2 and antioxidant response element expression to modulate hepatic disorders [35]. The same function as hawthorn, polyphenols-enriched extracts from *Camellia sinensis* (belonging to Theaceae) also modulated lipid homeostasis based on the upregulation of PPARα expression. The administration of the extract ameliorated the fructose-induced hypertriglyceridemia and the insulin-resistance in a fructose-induced hamster model [36].

Oxymatrine—a monosomic alkaloid isolated from *Sophora flavescens*—was found to decrease the liver lipid accumulation by histopathological detection in rats fed with high fructose diet (35% fructose, 35% starch, 10% fat, and 20% protein by energy) for 8 weeks. The research of its activity and mechanism revealed that oxymatrine decreased FAS activity and increased the carnitine palmitoyl transferase 1α (CPT 1α) activity. Additionally, oxymatrine treatment down-regulated the mRNA expression of SREBP-1, FAS, and ACC, and up-regulated the mRNA expression of PPARα, CPT 1α and acyl-CoA oxidase, resulting in the regulation of hepatic lipid metabolism [37]. Betaine, another alkaloid from common edible plants, has been proved effective in treating NAFLD in fructose-induced rat models by up-regulating hepatic expression of LXRα and PPARα with attenuation of the changes of their target genes, including SREBP-1c, ACCα, SCD-1, FAS, CPT I, and CPT II to alleviate hepatic lipid accumulation and fatty acid induced oxidation stress [38]. Thymoquinone—a bioactive benzoquinone isolated from *Nigella sativa* seed—was reported to ameliorate high fructose diet induced depletion of superoxide dismutase and prevent downregulation in hepatic mRNA of PPARα in high fructose diet induced rats to prevent metabolic syndrome [39]. Proanthocyanidin—a polyphenol isolated from grape seed—was reported to increase PPARα more effectively compared to metformin in high-fat-fructose-diet-induced hyperlipidemic rats to promote insulin action [40]. Curcumin was investigated to be useful in the modulation of oxidative stress status and inflammation cascades in rats on high fructose diets, by regulating the serum level of glucose, insulin, leptin, cholesterol, triglycerides, and the expression of NF-κB in hepatocytes [41].

In addition to natural plants, supplementation with dietary *n*-3 fatty acids from fish oil was capable of improving hepatic lipid metabolic response in rats treated with a high-fructose diet as well. The study showed an increase effect on hepatic PPARα gene expression and a decrease effect on gene expression of carbohydrate responsive element binding protein and FAS in rats fed fish oil-rich diets [42].

3.3. Inhibition of Inflammatory Pathways

Suffering from fructose-induced oxidation stress—an excess accumulation of intracellular ROS—will lead to triggering inflammation. Excessive intracellular ROS affects the NF-κB pathway via insulin receptor. Several natural products can inhibit the activation of the NF-κB pathway associated with JNK, inflammatory factors like TNF-α and interleukin-6 (IL-6), and insulin receptor substrate-1 (IRS-1) in hepatic inflammation.

Extracts from grape pomace contain relatively high amounts of polyphenols and dietary fiber. Supplementation with grape pomace and its extract altered high-fat-fructose diet-induced activation of JNK in Wistar rats, resulting in a recovery of insulin signaling cascade observed in liver tissue [43].

Extracts from the root of *Withania somnifera* (belonging to Solanaceae) have been confirmed to have anti-inflammatory, antitumor, antioxidant, immunomodulatory, and antistress activities. The latest study suggests that *W. somnifera* normalizes fructose-induced hyperglycemia in rats by reducing the increases of blood glucose, insulin, homeostasis model assessment for insulin resistance, IL-6 and TNF-α, thus alleviating inflammation and improving insulin sensitivity [44].

Isoorientin—a flavonoid isolated from several edible plants—remarkably ameliorated inflammation to inhibit hyperlipidaemia and liver injury by enhancing antioxidant enzyme activities and inhibiting inflammatory cytokine (TNF-α, IL-1, IL-6) secretion in high-fructose-induced obese mice [45]. Curcumin was also reported to attenuate insulin resistance through its anti-inflammatory and antioxidant effects. The administration of curcumin lowered expressions of TNF-α, C reactive protein, cyclo-oxygenase 2, and protein kinase C (PKC)θ, and prevented the activation NF-κB by preventing the degradation of inhibitor of nuclear factor κB (IκB)α in high fructose fed male Wistar rats [46]. (−)-Epicatechin—a flavanol abundant in many dietary plants—downregulated negative regulators such as PKC, IκB kinase, JNK, and protein tyrosine phosphatase 1B in the liver of high fructose-fed rats to mitigate fructose-induced metabolic disorders [47]. Geraniol—a monoterpene alcohol isolated from the essential oil of rose and lemon—prevented the increase of IL-1β and TNF-α in serum, as well as the increase of nitric oxide in liver, modulating fructose-induced inflammation and oxidative stress [48]. Astaxanthin—a xanthophyll carotenoid isolated from *Haemococcus pluvialis*—was reported to inhibit the phosphorylation of JNK1 and IκB-β, production of ROS, and nuclear translocation of NF-κB p65 in liver to alleviate inflammation in high fructose-fat diet-fed mice [49].

3.4. Improvement of Insulin Resistance

Liver is a major target tissue of insulin action. Over consumption of fructose stimulates hepatic lipogenesis, mitochondrial dysfunction and inflammation leading to deficiency of insulin sensitivity. Kinds of natural products mitigate insulin resistance through activating PI3K/Akt and AMPK pathways.

Flavonoids extracted from *Lomatogonium rotatum* (belonging to Gentianaceae) enhanced threonine-172 phosphorylation of AMPK and downregulated FAS mRNA expression and leptin levels in liver of high-fructose fed rats. The flavonoids may reduce lipid levels and prevent obesity by stimulating AMPK in hepatocytes [50]. Polysaccharides extracted from *Pleurotus eryngii* (belonging to Pleurotaceae) down-regulated fasting serum glucose and insulin concentrations by enhancing antioxidant activity in the liver of mice fed with fructose diet, with an amelioration of hepatic insulin resistance [51]. Terpenoids extracted from *Liriope platyphylla* (belonging to Liliaceae) altered the expression of Glut1, Glut3, and key proteins in the insulin signaling pathway in the liver of ICR mice treated with high fructose diets to mitigate insulin resistance. The hepatic expression level of Glut3 was down-regulated and Glut1 was up-regulated in response to the phosphorylation of the p38 protein mitogen-activated protein kinase (MAPK) and PI3-K/Akt signaling pathways [52].

Ferulic acid—a phenolic compound isolated from various fruits, vegetables, and some herbal medicines—reduced Glut2 expression by impairing the interaction between Glut2 gene promoter and transcription factors, including SREBP-1c, hepatocyte nuclear factor 1α (HNF1α), and HNF3β in fructose-induced type-2 diabetic rats to improve insulin sensitivity [53]. Resveratrol—a phenol compound isolated from many edible plants—reversed the hepatic triglyceride content in rats induced by high fructose corn syrup by promoting IRS, endothelial nitric oxide synthase, and sirtuin 1, and suppressing FAS and SREBP-1c expression to improve hepatic insulin resistance [54]. This activity was also reported to be partly related to the mRNA expressions of IL-6, IL-10, and IL-18, as well as inducible nitric oxide synthase, Nrf2, and PI3K [55]. In addition, genistein—an isoflavone of soybean used as a kind of phytoestrogen—improved the insulin-stimulated tyrosine phosphorylation of insulin receptor-β and IRS-1, phosphorylation of PI3K and Akt Ser473, and phosphorylation of AMPK Thr172. Significant decrease in IRS-1 serine phosphorylation and S6 kinase-1 Thr389 phosphorylation were observed in high-fat–high-fructose diet-induced mice to attenuate insulin

signaling [56]. Astaxanthin—a xanthophyll carotenoid isolated from *Haemococcus pluvialis*—was also reported to alleviate insulin resistance by modulating metabolic enzymes. Liver tissue from astaxanthin-treated mice fed with high-fat–high-fructose diet showed an increase effect on tyrosine phosphorylation and a decrease effect on serine phosphorylation. Astaxanthin increased the association between IRS 1/2 and PI3K and serine phosphorylation of Akt, and decreased fructose-induced serine kinases (JNK-1 and extracellular signal-regulated kinase-1), suggesting that the treatment promotes the IRS-PI3K-Akt pathway of insulin signaling by decreasing serine phosphorylation of IRS proteins [57].

4. Conclusions

This paper provided an overview of the current state of knowledge regarding the pathogenesis of fructose-induced NAFLD, and summarized natural products in the literature over the past years with a regulation effect on high fructose-induced NAFLD animal models. Interestingly, these active compounds exert their effects in multi-target manners. For example, phenolics mainly ameliorate NAFLD by affecting the level of SREBP-1c, FAS, SCD, ACC, PPARα, Nrf2, JNK, Glut2, IRS, and PI3K. Curcumin was reported to be effective in hepatic lipogenesis, mitochondrial dysfunction, inflammation, and insulin resistance in high fructose diet animal models. These natural products could provide new starting points in NAFLD new drug research and discovery.

5. Prospect

Clinical treatments of NAFLD chiefly includes surgical treatment and drug intervention. Bariatric surgery is one of the treatment options for NAFLD to regulate hepatic glucose and lipid metabolism [58], improve glucose, lipid profiles [59], and insulin resistance [60]. However, as with any major surgery, the procedure does carry some risks. Drug intervention is another method in NAFLD therapy. Metformin (dimethylbiguanide)—derived from active compound of *Galega officinalis* (Fabaceae) [61]—is an AMPK-activating molecule that has been used in the treatment of type-2 diabetes for more than 50 years [24]. It can decrease the levels of serum aminotransferases in patients with histologically confirmed NAFLD during the first 3 months of treatment [62]. However, the low response rate cannot meet the clinical requirements.

Obviously, it is urgent to develop therapeutic and preventive strategies for NAFLD. Natural products could provide new starting points in new NAFLD drug research and discovery. More and more researchers are paying attention to natural products to solve the emergent condition on NAFLD. According to studies over the latest years, effective natural products on fructose-induced NAFLD mainly focus on phenolics, flavonoids, alkaloids, and terpenoids. These compounds are absolutely different in chemical structures, but their targets may be similar to some degree. Therefore, it is difficult to ascertain how they could regulate the same target in NAFLD. Quantitative structure–activity relationship research methods can be applied in this field to provide scientific evidence. Although the activity of natural products is not stronger than that of synthetic compounds, it also supplies a potential source to develop better new drugs or dietary supplements for NAFLD.

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

Abbreviations

ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ACS	Acyl-CoA synthase
AMPK	Adenosine 5′-monophosphate (AMP)-activated protein kinase
ATGL	Adipose triglyceride lipase
CPT-1	Carnitine palmitoyl transferase 1
DGAT	Diacylglycerol acyltransferase
eNOS	Endothelial nitric oxide synthase
FAS	Fatty acid synthase
FFA	Free fatty acid
Fru	Fructose
Fru-1-P	Fructose-1-phophate
Glut5	Glucose transporter 5
GPAT	Glycerol-3-phosphate acyltransferase
HNF1α	Hepatocyte nuclear factor 1α
HSL	Hormone-sensitive lipase
IKK	Inhibitor of nuclear factor κB (IκB) kinase
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
JNK	c-Jun amino terminal kinase
LKB1	Liver kinase B1
LXRα	Liver X receptor α
MAPK	Mitogen-activated protein kinase
MCD	Malonyl-CoA decarboxylase
mTOR	Mammalian target of rapamycin
NAFLD	Nonalcoholic fatty liver disease
NF-κB	Nuclear factor kappa B
Nrf2	Nuclear factor-erythroid 2-related factor 2
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PPARα	Peroxisome proliferator–activated receptor α
PPARγ	Peroxisome proliferator activated receptor γ
ROS	Reactive oxygen species
SCD	Stearoyl-CoA desaturase
SREBP-1c	Sterol regulatory element binding protein 1c
TAG	Triglyceride
TNF-α	Tumor necrosis factor α
VLDL	Very low density lipoprotein

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Article

Metabolic Effects of Replacing Sugar-Sweetened Beverages with Artificially-Sweetened Beverages in Overweight Subjects with or without Hepatic Steatosis: A Randomized Control Clinical Trial

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Abstract: Objective: Addition of fructose to the diet of normal weight and overweight subjects can increase postprandial plasma triglyceride and uric acid concentration. We, therefore, assessed whether replacing sugar-sweetened beverages (SSB) with artificially-sweetened beverages (ASB) in the diet of overweight and obese subjects would decrease these parameters. Methods: Twenty-six participants of the REDUCS study, which assessed the effects of replacing SSB by ASB over 12 weeks on intra-hepatocellular lipid concentration, were included in this sub-analysis. All were studied after a four-week run-in period during which they consumed their usual diet and SSBs, and after a 12-week intervention in which they were randomly assigned to replace their SSBs with ASBs (ASB arm) or to continue their usual diet and SSBs (control arm, CTRL). At the end of run-in (week 4) and again at the end of intervention (week 16), they took part in an 8.5 h metabolic investigation during which their plasma glucose, insulin, glucagon, lactate, triglyceride (TG), non-esterified fatty acids (NEFA), and uric acid concentrations were measured over a 30 min fasting period (−30–0 min), then every 2 h over 480 min. with ingestion of standard breakfast at time 0 min and a standard lunch at time 240 min. Breakfast and lunch were consumed together with a 3.3 dL SSB at week 4 and with either an ASB (ASB arm) or a SSB (CTRL arm) at week 16. After analyzing the whole group, a secondary analysis was performed on 14 subjects with hepatic steatosis (seven randomized to ASB, seven to CTRL) and 12 subjects without hepatic steatosis (six randomized to ASB and six to CTRL). Results: Ingestion of meals increased plasma glucose, insulin, glucagon, lactate, and TG concentrations and decreased NEFA concentrations, but with no significant difference of integrated postprandial responses between week 4 and week 16 in both ASB and CTRL, except for a slightly decreased glucagon response in ASB. There was, however, no significant postprandial increase in uric acid concentration in both arms. In the secondary analysis, replacing SSBs with ASBs did not significantly change postprandial TG and uric acid concentrations irrespective of the presence or not of hepatic steatosis, Conclusions: In overweight, high SSB consumers, replacing SSBs with ASBs during 12 weeks did not significantly alter post-prandial TG and uric acid concentration, in spite of the lower energy and fructose content of the meals. These effects were globally the same in subjects without and with hepatic steatosis.

Keywords: intrahepatocellular lipid concentration; cardiovascular risk factors; fructose; hypertriglyceridemia

1. Introduction

There is presently much concern that high fructose consumption may play a causal role in the development of metabolic and cardiovascular diseases [1–4]. Furthermore, the unique propensity of fructose to stimulate hepatic de novo lipogenesis (DNL) has made it a prime suspect for the development of non-alcoholic fatty liver disease (NAFLD), although direct evidence remains limited [5,6]. The REDUCS study is a small controlled randomized clinical trial assessing the effects of replacing sugar-sweetened beverages (SSBs) by non-caloric, artificially-sweetened beverages (ASBs) on intra-hepatocellular lipid concentrations (IHCL) in high-SSB consumers with overweight or obesity. Replacing SSB with ASB was the sole intervention, and, although subjects were advised not to change their usual diet, food intake was otherwise left ad libitum. We have reported elsewhere that ASB significantly reduced IHCL compared to control [7]. There was, however, no significant change in body weight, nor on any of the other fasting metabolic parameters. In this setting, we hypothesized that a reduction in IHCL was possibly an early marker of negative energy balance.

In addition to promoting excess energy intake and body weight gain, several investigators have raised concern that fructose-induced DNL may be responsible for dyslipidemia (mainly fasting and postprandial hypertriglyceridemia) [8], tissue lipotoxicity [4], hyperuricemia, and insulin resistance [9] and, hence, that dietary fructose may exert adverse health effects even in the absence of an excess energy intake [10,11]. A secondary outcome of the REDUCS study was to evaluate whether replacing SSBs with ASBs would have beneficial effects on postprandial metabolic homeostasis and, more specifically, on triglyceride and uric acid concentrations. To this purpose, we monitored the plasma concentration of various hormones and metabolites over an 8.5 h period during which participants received one standardized breakfast and one standardized lunch, together with one SSB serving at the end of a four-week run-in period, and either one SSB or one ASB serving according to randomization at the end of the subsequent 12-week intervention.

It has also been suggested that dietary fructose may exert larger detrimental effects in insulin-resistant than in insulin-sensitive subjects [12], suggesting a potential synergic effect. Since NAFLD is strongly associated with insulin resistance [13], and since IHCL have been proposed to be even more closely associated with an increased cardiometabolic risk than visceral body fat mass, we also ran a secondary analysis to assess the effects of replacing SSBs with ASBs in participants with or without hepatic steatosis.

2. Methods

2.1. Subjects' Inclusion

Male and female subjects with BMI > 25 kg/m^2 and a habitual daily consumption of two or more servings (22 oz or 660 mL per day or more) of SSBs (defined as carbonated soft drinks and sugar sweetened iced tea) were eligible for this study. Criteria of inclusion were: having maintained a stable body weight (less than 4 kg variation) for the past 18 months; low to moderate physical activity (up to four 30 min-sessions of exercise per week); not being presently attempting to lose weight; and having no known disease. Criteria of exclusion were: any current drug treatment (except oral contraceptive agents); recent or planned pregnancy; consumption of alcohol more than 10 g per day; being on a special diet and; having contraindication for magnetic resonance evaluation.

Recruitment was done by advertising in the local press and through social networks. The screening, enrollment, randomization, and follow-up of study participants are depicted in Figure 1. Potential participants were initially screened by a phone interview. Seventy-five potential participants were invited for a screening visit including a medical history and a physical examination in order to ensure good physical health. Of 35 subjects found eligible, two quit the study before the first metabolic test, and MRS measurements could not be obtained in another two. The experimental protocol was approved by the Ethical Committee for Human Research of the Canton de Vaud, Switzerland, and registered on clinicaltrials.gov (NCT 01394380). All participants provided an informed, written consent

at inclusion. Of the 35 participants initially randomized, five dropped out after the run-in period. The twenty-six subjects (13 in ASB and 13 in CTRL), having completed all metabolic investigations, were included in this secondary analysis.

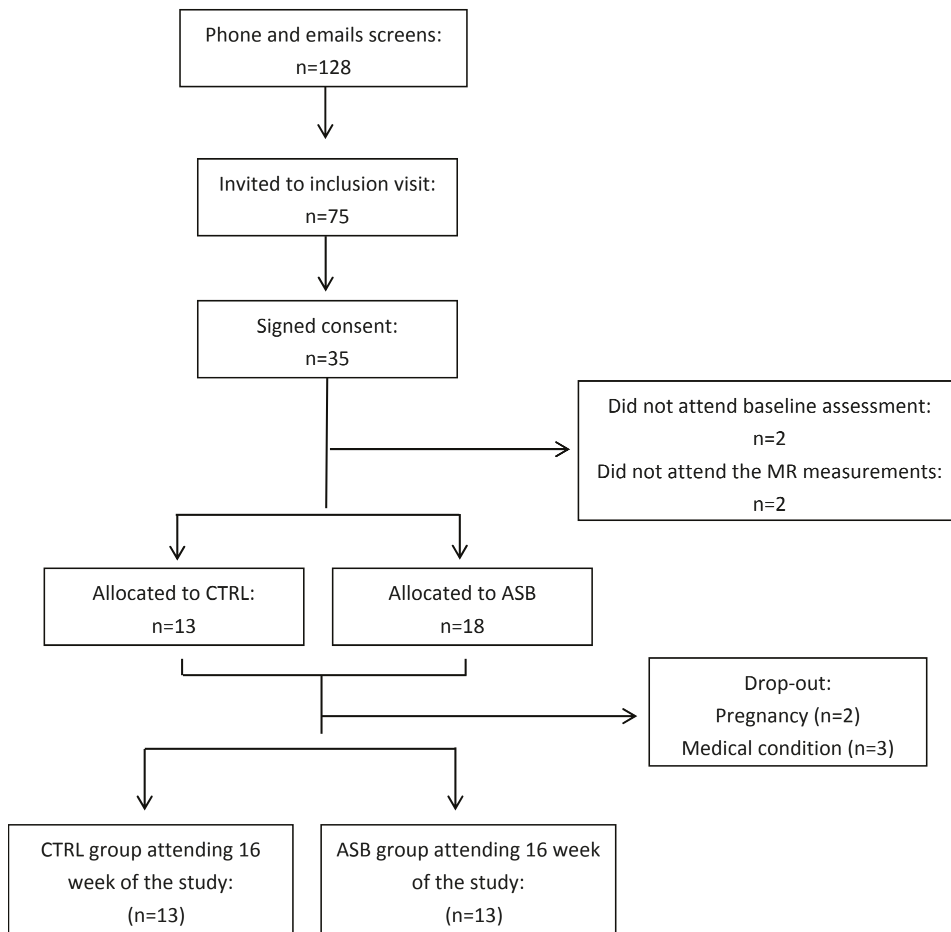

Figure 1. Eligibility, enrollment, randomization, and follow-up of study participants.

After inclusion, all 26 subjects (13 males, 13 females, age range: 20–43 years) entered a four-week run-in period, during which they consumed SSBs and performed their usual daily occupations and physical activity as usual (week 1–week 4). They received every week the number of SSB servings corresponding to their usual weekly consumption, and were asked to return the empty packages on their next weekly visit as a measure of compliance.

During the fourth week, they had to wear an actimeter (DIGI-WALKER SW-2000, Yamax, Japan) and to fill detailed records of their food and beverage intake during two non-consecutive days (working days). For this evaluation, they obtained photographs of each plate they consumed on their mobile phone. A calibration device (pen) was positioned next to the plate to allow subsequent estimation of the quantity of foods present on the plate. At the end of this week, they also had a 60–90 min visit with a nutritionist to verify and discuss their dietary records and to estimate portion sizes.

On day 27, they reported to the Magnetic Resonance Research Center at the Inselspital Bern, where their intra-hepatocellular lipid (IHCL) concentration and visceral fat volume (VAT) were measured. Between day 27 and 28, they performed a 24-h urine collection.

On day 28, subjects were asked to come to the Centre of Clinical Research (CRC) at 0700 in the fasting state, and underwent a metabolic test with the ingestion of two test-meals.

Thereafter, subjects were randomly assigned to consume either SSBs or ASBs for three months. Randomization was stratified by sex. Their food intake and non-sweetened beverages consumption was left ad libitum during this period. The number of either ASB or SSB servings corresponding to their SSB consumption during the run-in period was distributed on a weekly basis, and participants were asked to consume only these beverages, to abstain from any other beverage except for water, tea, or coffee, and to return empty beverage packs at their next visit as a monitoring of compliance

On weeks 10 and 16, they had their food intake estimated from two-day food records, their physical activity was monitored with an actimeter, and their 24-h urines were collected. These data are, however, not presented here. All of the measurements done at week 4 (end of run-in) were obtained again at week 16 (end of intervention). The study flow chart is shown in Figure 2.

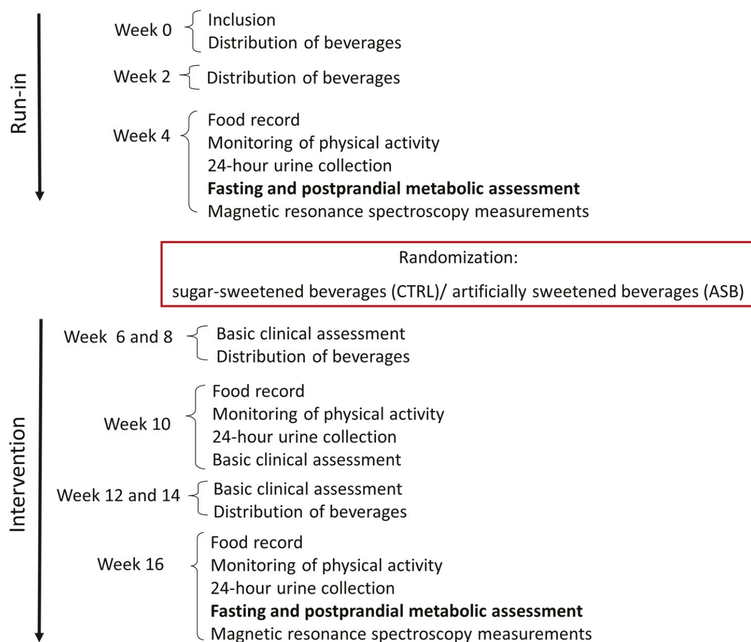

Figure 2. Experimental protocol. Every subject was asked to continue her/his usual diet and physical activity throughout the study; during the initial study, all subjects received SSBs according to their usual consumption (run-in). Thereafter, subjects were randomized to receive either ASB (ASB group) or SSBs (CTRL group) for the next 12 weeks (intervention). Throughout the study, subjects visited the clinical research center every two weeks to collect beverages, return empty cans consumed at home, and for anthropometric measurements and clinical chemistry laboratory (basic clinical assessment). Metabolic investigations were done at week 4 and 16. They also had food their food intake monitored every six weeks.

2.2. Metabolic Tests

Each subject underwent a detailed metabolic investigation at the end of the run-in period and at the end of the 12-week intervention. For these investigations, subjects reported at about 7 a.m. to the Clinical Research Center of Lausanne University Hospital. They were fasted since 10:00 p.m. the day before, and traveled to the hospital by public transportation, with minimal physical activity involved. At their arrival at the Clinical Research Center, they were asked to void their bladder, and their urine was discarded. Urine was thereafter collected until the end of the test for the determination of the urinary urea nitrogen excretion rate. Subjects were weighed and the body composition was assessed by bio-electrical impedance (Imp. Df. 50; ImpediMed, Pinkenba, Australia). They were then transferred to a bed, where they remained lying until the end of the test. A catheter was inserted in a vein of the right arm, which was subsequently used for blood collection.

After a 30 min period of baseline measurements, two test-meals, each containing 15% protein, 30% fat, and 55% carbohydrate, were administered at time 0 and time 240 min. Breakfasts and lunches provided 25% and 35% of the estimated 24-h energy requirements, respectively (equal to basal energy expenditure calculated with the Harris Benedict equation times a physical activity level of 1.5). The breakfast was composed of one turkey breast sandwich with butter and of one yogurt with jam. The lunch was composed of one egg, cheese and butter sandwich, one plain yoghurt, and dried apple slices. At week 4 (end of run-in), all participants drank one 3.3 dL SSB (35 g sugar, 139 kcal) with each meal. Macronutrients and energy content of test meals are shown in Table 1. At week 16, they drank either SSBs or ASBs with meals according to the treatment arm. Overall energy intake was, therefore, higher for participants in the SSB arm (ca. 70% their 24-h energy requirements) than in the ASB arm (ca. 60%). Blood samples were collected every 120 min until 480 min after ingestion of the breakfast.

Table 1. Nutrient content of the test meals.

	Week 4 Test Meals	Week 16 Test Meals	
		ASB	CTRL
Breakfast			
Energy (kcal)	748	658	762
Starch (g)	50	53	51
Sucrose (g)	49	15	49
Fat (g)	20	22	21
Protein (g)	23	25	23
Lunch			
Energy (kcal)	981	895	1009
Starch (g)	67	71	70
Sucrose (g)	56	22	57
Fat (g)	28	30	29
Protein (g)	31	33	32

2.3. Analytical Procedures

Plasma was immediately separated from blood cells by centrifugation at 1230× *g* for 10 min at 4 °C, and plasma aliquots were stored at −20 °C. Plasma metabolites (glucose, TG, non-esterified fatty acids (NEFAs), uric acid, and lactate) and urinary urea were measured by enzymatic methods (Randox Laboratories, Crumlin, UK). Insulin and glucagon were assessed by radioimmunoassay (Millipore, Billerica, MA, USA). Plasma fructose concentrations were measured by gas chromatography-mass spectrometry (GC-MS) as reported elsewhere [14].

IHCL content was measured by 1H-MRS on a clinical 3 Tesla MR system (TIM Trio, Siemens Medical, Germany) and VAT was determined using T1-weighted images of the abdomen, as previously described [15].

Total energy, carbohydrate, fat, and protein intakes were calculated from two-day food records with the Prodi 5.8 software (Nutri-Science Gmbh, Stuttgart, Germany). Each participant's daily nutrient, energy, and beverage intakes were calculated by taking the average of two records obtained on two weekdays. Liquid sugar intake was calculated as the sum of sugars from SSBs, milk, coffee, tea, and alcoholic beverages. Sugars outside SSB were calculated by subtracting SSB sugars from the total sugar intake.

2.4. Statistical Analysis

All values are expressed as means $\pm$ standard error of the mean (SEM). Data normality was checked with the Shapiro-Wilk test. Non-normally distributed data were log-transformed before statistical analysis. Baseline characteristics between ASB and SSB groups were assessed by Student's unpaired *t*-tests.

In a first calculation, we analyzed the global postprandial responses on the whole group of 26 subjects. For this purpose, we calculated, for each measured variable, the incremental area under the curve (iAUC) between time 0 min (i.e., immediately before breakfast) and time 480 min (i.e., 8 h after breakfast and 4 h after lunch); for these calculations, the mean of three fasting values obtained at time -30, -15, and 0 min were subtracted from postprandial values measured at time 120, 240, 360, and 480 min. When iAUC was significantly different from zero (which was the case for all variables except uric acid), a two-way ANOVA with interaction was performed to assess significant differences between the beginning and the end of intervention on the whole sample (effect of time), significant differences between intervention arms (intervention) and interactions between time and intervention.

In a second calculation, we performed a separate detailed kinetic analysis on all outcomes in the subgroups of 12 participants (three males, nine females, mean BMI 28.6 $\pm$ 1.1, age range 21–39 years) without hepatic steatosis (defined as IHCL < 5.5%), and in, a subgroup of 14 subjects (10 males, four females, mean BMI 32.8 $\pm$ 1.3, age range 20–43 years) with hepatic steatosis (IHCL > 5.5%). Comparisons were done by two-way ANOVA for repeated measures with interaction, with time (week 4 and week 16) and intervention (ASB or CTRL) as independent variables. Specific time-points with significant differences were identified with paired-t-tests when ANOVA showed a significant effect of intervention, or a significant interaction between time and intervention.

All statistical calculations were performed with Stata 10 (Stata, College Station, TX, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Anthropometric Variables, Dietary Intake, Fasting Plasma Metabolic Markers, and IHCL

Body weight, body composition, and fasting metabolic parameters at the end of the run-in period were already reported for all participants ($n = 27$) and did not differ significantly between groups. Fasting concentration of IHCL was somewhat larger in the CTRL group due to two subjects having IHCL > 325 mmol/L, however. The effects of intervention on spontaneous food intake have also been reported. Total energy intake decreased by 28%, and total sugar intake by 68% between week 4 and week 16 in the ASB arm, but did not change in CTRL. The effects of intervention on the main study outcome, IHCL, has been reported for all 27 participants [7]. IHCL decreased significantly in the ASB arm, but not in the CTRL arm, while body weight (90.1 vs. 91.0 in CTRL, 93.9 vs. 92.5 in ASB) did not change significantly in either group. There was also no statistically significant difference for total body fat mass, visceral fat volume, nor for all measured fasting metabolic substrate and hormone concentrations [7].

3.2. Postprandial Metabolic Responses

As a first step we analyzed postprandial time courses of each individual variables in all subjects. Ingestion of breakfast and lunch increased plasma glucose, fructose, lactate, triglyceride, uric acid, insulin, and glucagon concentrations, and decreased plasma NEFA concentrations. There was, however, no significant effect of time or intervention, and no significant time × intervention interaction. Data have not been displayed as figures for the whole group since they will be presented below in each subgroup. We then specifically assessed the postprandial effect of meal ingestion by reducing each variable to a single value, i.e., the incremental area under the curve (iAUC) cumulated between the beginning of breakfast to 4 h after ingestion of lunch and searching for effects of time (week 4 vs. week 16), treatment (CTRL vs. ASB), and time × treatment interaction (Table 2). This procedure assesses, globally, the responses of plasma metabolite concentrations induced by breakfast and lunch. Plasma glucose, insulin, lactate, and triglyceride concentrations increased after meals, resulting in large, positive iAUC, while plasma NEFA concentrations decreased, resulting in negative iAUCs. There was no significant effect of intervention, nor time × intervention interactions for any of these variables, but there was a trend for time effect (p = 0.06) and for a time × intervention interaction (p = 0.08) for lactate. Fructose and glucagon both increased slightly after meals, with a significant effect of intervention for glucagon and a significant time × intervention interaction for fructose, which decreased in the ASB arm and increased in the CTRL arm. iAUC for uric acid were not significantly different from zero, indicating no significant postprandial change.

We then ran the same analysis separately in the subgroups of participants without and with hepatic steatosis separately. Participants with hepatic steatosis had more cardio-metabolic risk factors, i.e., higher BMI, VAT, and Homeostasis Model Assessment of Insulin resistance (HOMA) index, plasma triglyceride and cholesterol concentrations, uric acid concentrations, and lower HDL-cholesterol concentrations. They also had larger decreases of IHCL with ASB [7].

Postprandial plasma glucose, insulin, lactate, and glucagon concentrations in participants without hepatic steatosis are shown in Figure 3. Peak plasma insulin and lactate concentrations after breakfast were significantly lower at week 16 than at week 4 in the ASB arm, but not in the CTRL arm. Postprandial plasma TG, NEFA, uric acid, and fructose are shown in Figure 4. Except for lower postprandial fructose concentrations after intervention in the ASB arm, these parameters were not significantly changed after intervention.

Postprandial plasma glucose, insulin, lactate, and glucagon concentrations in participants with hepatic steatosis are shown in Figure 5. Peak lactate concentrations after breakfast and lactate concentration at 480 min were significantly lower at week 16 than at week 4 in the ASB arm, but not in the CTRL arm.

Postprandial plasma TG, NEFA, uric acid, and fructose concentrations are shown in Figure 6, and were not statistically different at week 4 and at week 16, except for a slightly lesser suppression of NEFA and higher peak fructose concentrations in the ASB arm.

4. Discussion

The present worldwide epidemics of obesity and of related metabolic diseases are the consequence of an excess energy intake relative to energy expenditure, largely driven by environmental factors [16,17]. There is no question, based on the first law of thermodynamic, that obesity is associated with storage of large amounts of energy within body fat and, hence, this blunt statement holds true whether excess energy intake results of altered homeostatic mechanisms or of factors merely linked to modern lifestyle. Sugar-sweetened beverages and fruit juice consumption worldwide average 0.74 serving per day for the whole population. It shows some between-country variations, with an average consumption of 0.76 servings per day in high-income countries compared to 0.46 in low-income countries. Within each country, it also shows large inter-individual variations, mainly according to age and gender, with slightly higher values observed in males and in young adults [18].

Table 2. Postprandial incremental area under the curve (iAUCpp) of metabolites and hormones concentrations in all participants. Values are expressed as mean ± 1 SEM.

	ASB (n = 7 ♂, 6 ♀)		CTRL (n = 6 ♂, 7 ♀)		p Values (2-Way ANOVA)		
	Week 4	**Week 16**	**Week 4**	**Week 16**	**Time**	**Intervention**	**Time × Intervention**
Lactate iAUCpp (mmol/L × 480 min)	200.1 ± 23.2	153.0 ± 24.7	161.3 ± 38.7	175.6 ± 33.8	0.3503	0.0586	0.0872
Glucose iAUCpp (mmol/L × 480 min)	645.3 ± 27.6	700.6 ± 62.7	644.2 ± 112.7	645.1 ± 69.4	0.5045	0.5034	0.5192
NEFA iAUCpp (mmol/L × 480 min)	−144.3 ± 22.2	−129.0 ± 20.5	−132.9 ± 31.4	−106.4 ± 28.4	0.2240	0.8856	0.7383
Glucagon iAUCpp (pg/mL × 480 min)	5291.1 ± 1052.0	4684.6 ± 855.8	4367.1 ± 659.5	5248.2 ± 1152.2	0.8551	0.0148	0.3273
Insulin iAUCpp (μU/mL × 480 min)	23,502.9 ± 3246.0	17,734.2 ± 2772.0	21,216.0 ± 4436.4	21,348.0 ± 4123.9	0.1380	0.2571	0.1213
Triglycerides iAUCpp (mmol/L × 480 min)	218.9 ± 23.7	189.5 ± 47.2	214.2 ± 46.7	240.1 ± 67.0	0.9506	0.6001	0.3338
Fructose iAUCpp (μM/L × 480 min)	24.0 ± 2.1	19.12.4	19.5 ± 4.1	21.3 ± 5.0	0.2231	0.6329	0.0456
Uric acid iAUCpp (mmol/L × 480 min)	27.1 ± 25.9	16.2 ± 11.2	22.6 ± 39.8	28.5 ± 35.9	NA [1]	NA [1]	NA [1]

[1] NA: not applicable: ANOVA was not performed because iAUCpp were not significantly different from 0.

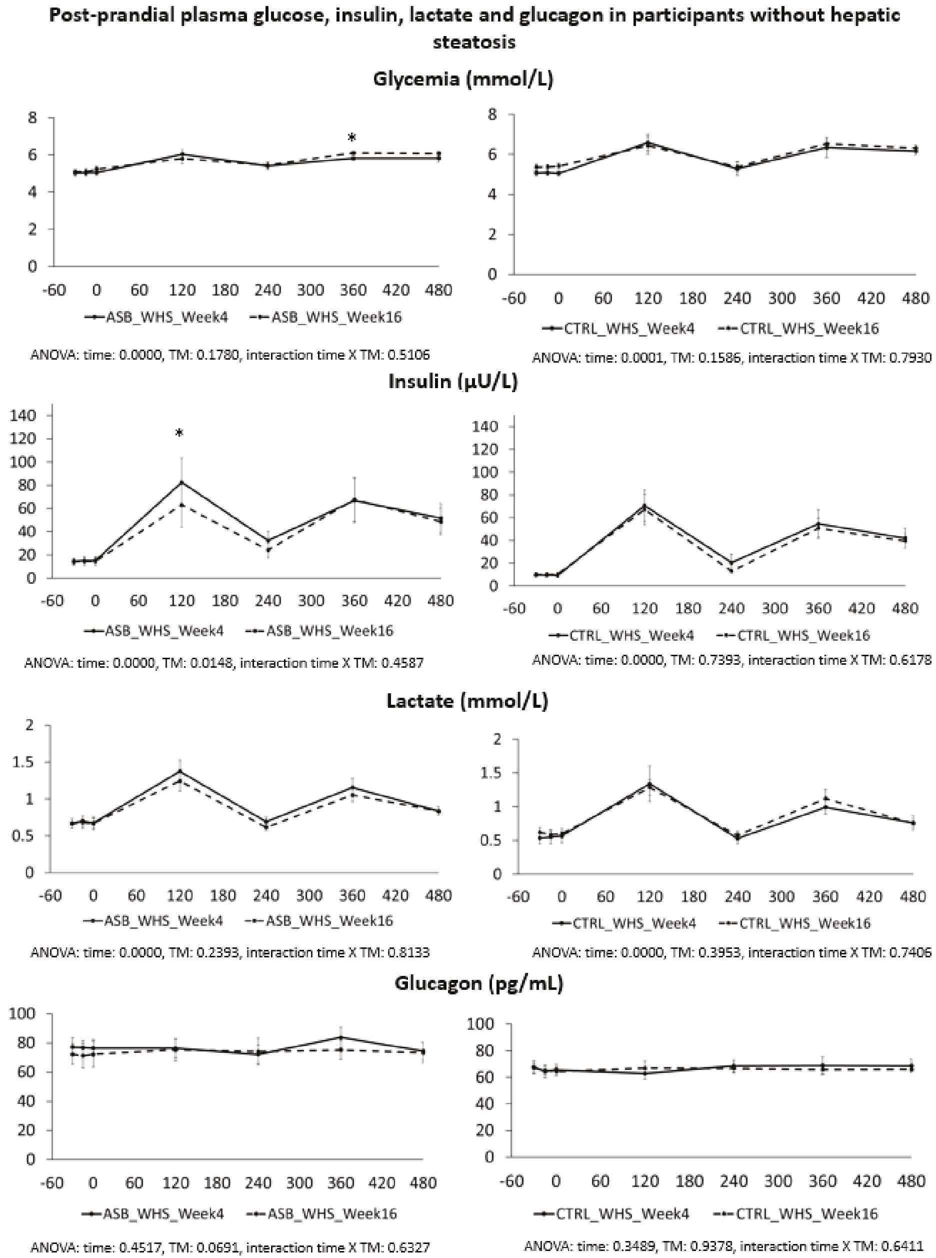

Figure 3. Plasma concentrations of glucose, insulin, lactate and glucagon in participants without hepatic steatosis. Data are expressed as mean ± SEM.; solid lines correspond to week 4, dotted lines to week 16. Results for ASB arm are shown on the left part, and for CTRL arm on the right part of the graph. Statistics (two-way ANOVA for repeated measurements with interaction) are reported at the bottom of each graph.

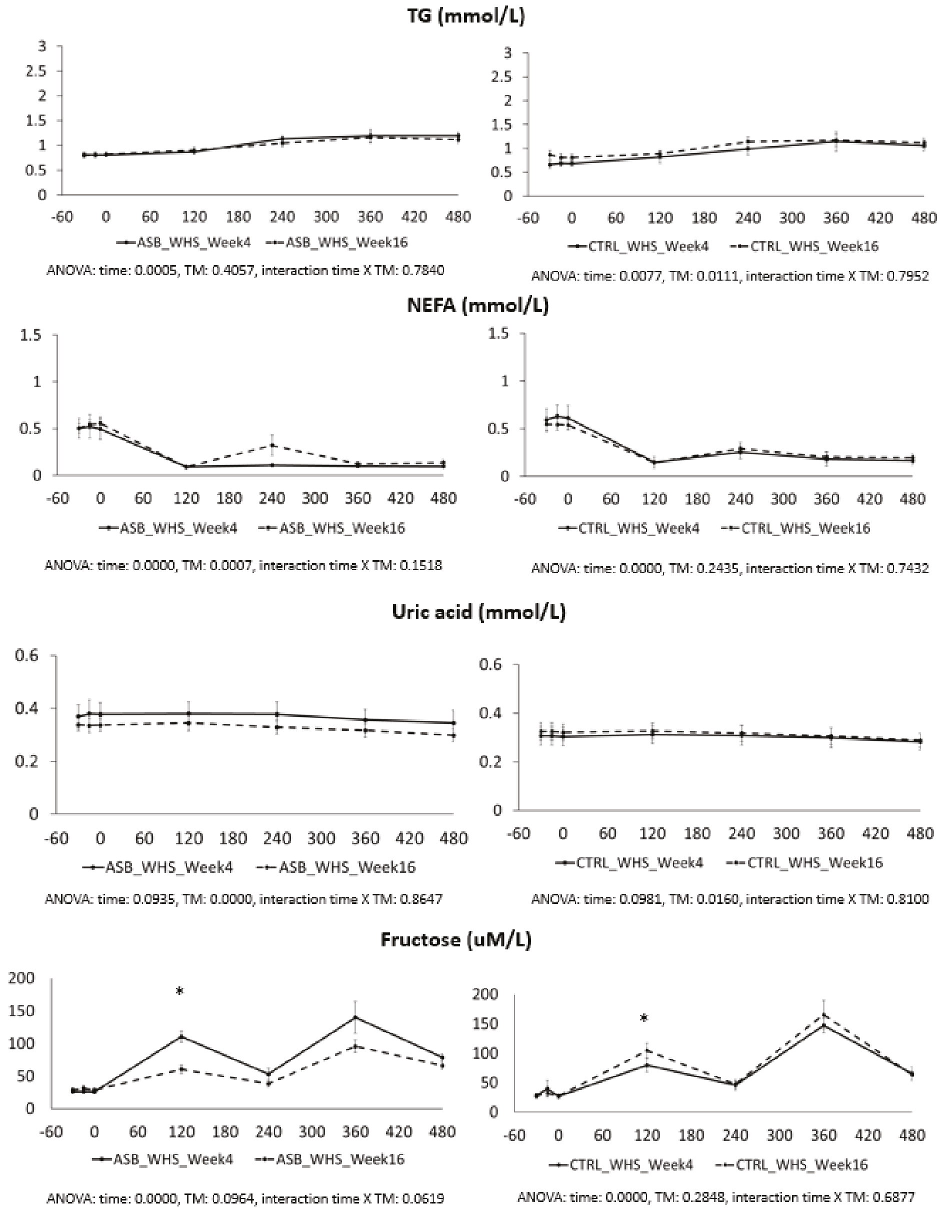

Figure 4. Plasma concentrations of TG, NEFA, uric acid, and fructose in participants without hepatic steatosis. Data are expressed as mean ± SEM.; solid lines correspond to week 4, dotted lines to week 16. Results for ASB arm are shown on the left part, and for CTRL arm on the right part of the graph. Statistics (two-way ANOVA for repeated measurements with interaction) are reported at the bottom of each graph.

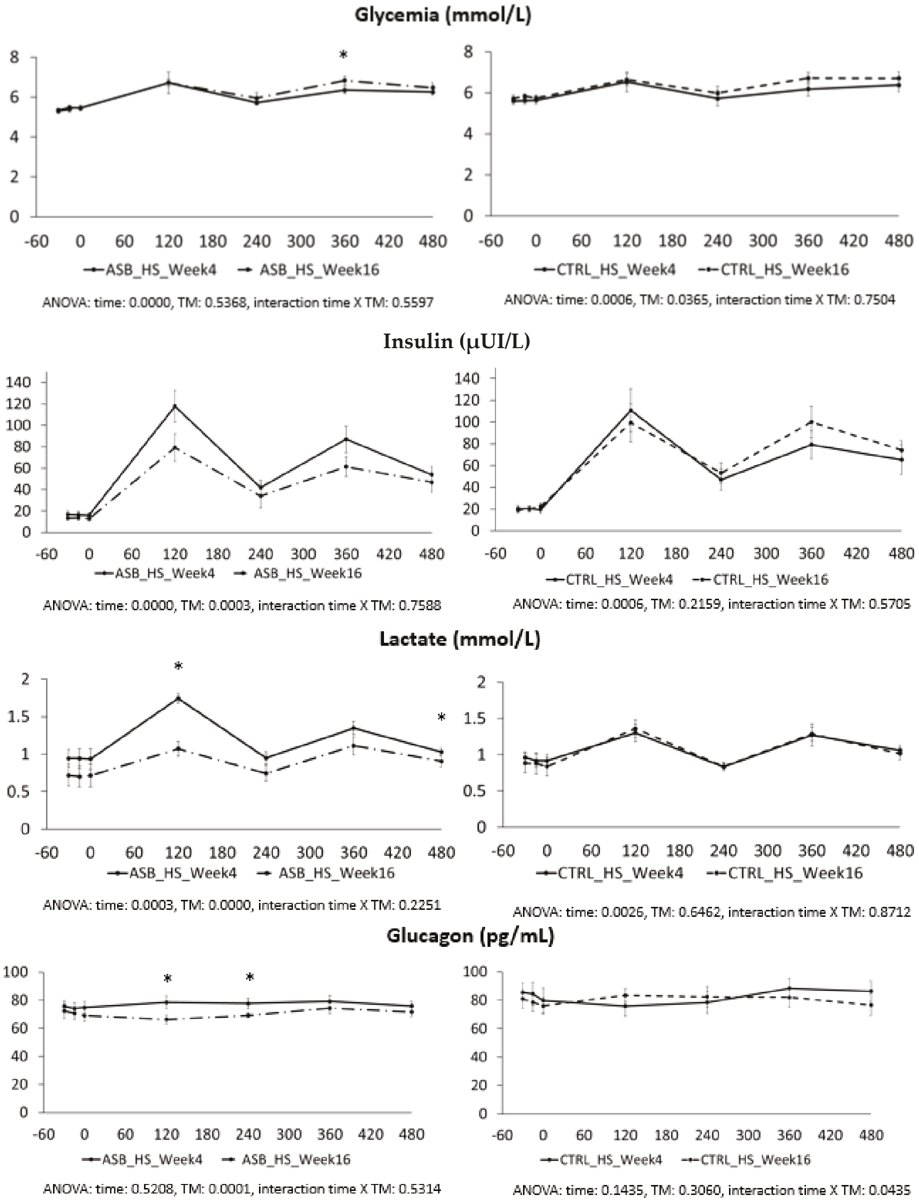

Figure 5. Plasma concentrations of glucose, insulin, lactate and glucagon in participants with hepatic steatosis. Data are expressed as mean ± SEM.; solid lines correspond to week 4, dotted lines to week 16. Results for ASB arm are shown on the left part, and for CTRL arm on the right part of the graph. Statistics (two-way ANOVA for repeated measurements with interaction) are reported at the bottom of each graph.

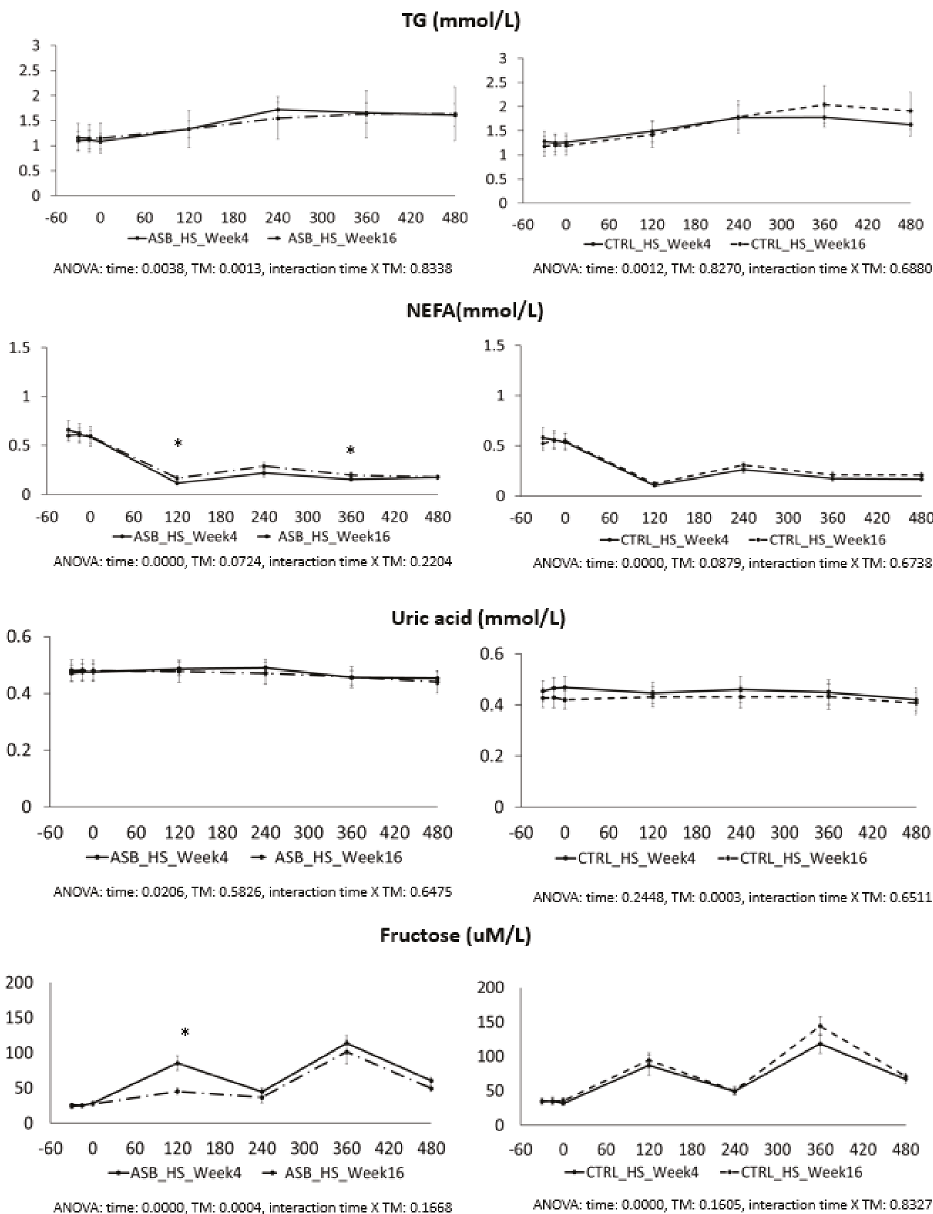

Figure 6. Plasma concentrations of TG, NEFA, uric acid, and fructose in participants with hepatic steatosis. Data are expressed as mean ± SEM.; solid lines correspond to week 4, dotted lines to week 16. Results for ASB arm are shown on the left part, and for CTRL arm on the right part of the graph. Statistics (two-way ANOVA for repeated measurements with interaction) are reported at the bottom of each graph.

SSBs, more than other energy-dense foods, have been proposed to be an ominous threat to body weight control and metabolic health for two main reasons. First, SSBs contain energy, yet their intake is triggered either by thirst, i.e., by physiological stimuli regulating body fluid homeostasis, or by social occasions. As such, SSB may escape the normal homeostatic mechanisms by which solid foods inhibit food intake [19]. Second, SSBs provide large amounts of mono- or disaccharides, of which approximately half is fructose. This monosaccharide first converts into glucose, lactate, or fat through the actions of specific enzymes located in splanchnic organs before being metabolized by extra-hepatic tissue [20]. There is, therefore, a concern that a high fructose intake, whether from SSBs or from "solid" foods, may impair blood glucose and lipid homeostasis by excessively stimulating gluconeogenesis and DNL even in the absence of excess energy intake. In support of this hypothesis, healthy volunteers had increased hepatic DNL and blood lipids when fed a high-fructose weight-maintenance diet [11,21]

The REDUCS study was designed to assess the effects of SSBs reduction on IHCL, but not on energy intake. Furthermore, it was of too short duration to assess the effects on body weight. As already discussed elsewhere [7] it, however, decreased intrahepatic fat concentration, which may be an early marker of a negative energy balance. In addition to the measurement of IHCL as the primary study outcome, the experimental protocol included a detailed assessment of postprandial metabolism after ingestion of breakfast and lunch at the end of the run-in period, and again at the end of the intervention [7]. All participants had consumed their usual diet with SSBs during the run-in period, and received breakfasts and lunches, each ingesting one SSB together with both lunch and breakfast. Breakfast, lunch, and SSBs together accounted for about 70% of 24-h energy requirements, i.e., corresponded to the usual energy consumed with these meals in a typical European diet. After intervention, participants of the CTRL arm continued on their usual SSB-containing diet for 12 weeks, and received the same breakfast and lunch together with SSBs. In contrast, the participants in the ASB arm had consumed an SSB-reduced, possibly hypocaloric diet during the preceding 12 weeks, and received the same breakfasts and lunches as after run-in, i.e., providing ca. 60% of the calculated energy requirement and ASBs containing no calories. Comparing the metabolic profile in the ASB vs. CTRL groups at week 16, therefore, assesses both the effects of a chronic fructose-reduced diet vs. a chronic high fructose diet, and the acute effect of a fructose-reduced hypocaloric meals vs. a fructose-containing meal.

Based on the proposal that fructose has severe adverse metabolic effects of its own, i.e., independent of effects on energy balance or body composition, one would have expected significant alterations of postprandial metabolic parameters. The effects of intervention on fasting metabolic markers have already been reported for the 27 participants of the study [7], and were not different for the 26 subjects included in the present analysis. Our present data further support our initial report. Participants in the ASB group consumed, on average, 65% less total sugar than participants in the CTRL group over 12 weeks, but there was no significant difference in postprandial TG and uric acid concentrations. This is particularly striking since test-meals contained the same amount of carbohydrate, fat, and protein from "solid" foods in both arms, but an additional 120 kcal and 15 g fructose with SSB in CTRL arm only. The global effect of ASB on glucose homeostasis is most likely under-evaluated by our data since we only obtained blood samples 2 and 4 h after ingestion of breakfast and lunch, while ASB most likely led to maximal reductions of glucose, lactate, and insulin between 30 and 90 min after meal ingestion.

Based on several reports documenting the occurrence of fasting and postprandial hypertriglyceridemia in subjects fed controlled high-fructose diets [22,23] we had expected that reduction of SSBs' consumption would come along with lower postprandial TG concentrations. In contrast with early postprandial glucose and insulin responses, plasma TG concentrations increase slowly over several hours [14,23], with a progressive increase between post-breakfast and post-lunch responses [22]. This pattern of TG response was, therefore, adequately assessed by collecting blood samples at two hours intervals. We, however, did not observe any significant decrease in the ASB arm, suggesting that neither the reduction of total sugar intake over the 12 previous weeks,

nor the consumption of a single SSB together with meals, had much impact on postprandial TG concentrations. The discrepancy may be due the fact that supplementation studies [22,23] had used high, supraphysiological fructose doses. Our present data may simply indicate that consumption of one standard 3.3 dL SSB serving with a standard meal provides too low a fructose load to induce statistically and clinically relevant effects on these parameters.

We also expected that uric acid concentration would be decreased in the ASB arm due to reduced fructose intake. Fructose is well known to increase hepatic uric acid synthesis [24] and may decrease renal uric acid excretion [25,26]. As for TG, we did not observe any significant changes in uric acid concentration in the ASB arm. There are, however, many factors which significantly impact uric acid concentrations, including genetic factors, dietary factors, or alcohol intake. One may, therefore, hypothesize that fructose contribution to uric acid concentrations was relatively small. Alternatively, it is possible that SSB reduction induced a negative energy balance which did not significantly impact on body weight due to the short duration of intervention, and that a reduction in uric acid from SSB reduction was balanced by an increased uric acid release from weight loss in this group. A recent study also reported no effects of replacing SSBs with pure glucose- or pure fructose-drinks during four weeks in Hispanic American adolescents with hepatic steatosis. Plasma TG concentration did not increase, and were even non-significantly decreased by ca. 30%, when SSBs were replaced by pure fructose drinks. In contrast, there was a significant reduction of plasma C-reactive proteins and oxidized low-density lipoprotein when SSBs were replaced by glucose, but not fructose, drinks, still suggesting that fructose was associated with adverse metabolic effects [27]. In contrast, another recent study reported significant reductions of fasting and postprandial plasma glucose insulin and triglyceride concentrations when added sugars were replaced with isocaloric amounts of starch over nine days in Hispanic and African American children [28]. This was a non-controlled study, however, and participants lost, on average, 1 kg body weight over the one-week intervention. It is, therefore, highly likely that these effects were mainly due to hypocaloric feeding; this rapid weight loss was also likely of origin of the increase in plasma uric acid concentration reported in this study [28].

We also hypothesized that fructose may exert more deleterious metabolic effects in insulin-resistant, than insulin-sensitive, subjects. In this regard, it is of interest that the study participants with hepatic steatosis were insulin resistant, but had higher plasma TG and uric acid concentrations than those without hepatic steatosis. Even in this subgroup, however, postprandial plasma TG and uric acid did not decrease after 12 weeks of intervention with sugar/fructose restriction. This suggests that factors others than the mere intake of SSBs play a major role in their hypertriglyceridemia and hyperuricemia. One may argue that the aforementioned studies were of too short a duration to observe the full range of beneficial effects of SSB reduction. However, while many intervention studies in which fructose or sugar were added to the usual daily intakes of participants documented significant increases in plasma triglycerides [29,30], few interventions involving the reduction of dietary sugar over several months or years have reported on plasma lipids. One such study, which had previously reported a lower BMI increase in children receiving ASBs than in controls receiving SSBs [31], has recently reported published data related to the study's secondary outcomes: in spite of a two-year intervention, there was also absolutely no effect of replacing SSBs with ASBs on fasting blood triglyceride concentrations [32].

SSB reduction had no significant effect on postprandial metabolic risk factors, yet significantly reduced IHCL, which was the primary endpoint of the study. Due to its two-step metabolism, which includes a conversion of its carbons into glucose, lactate, and fatty acids, fructose affects muscle and adipose metabolism through mechanisms distinct from glucose or dietary fat. There is growing evidence that subtle alterations of inter-organ substrate fluxes, leading to ectopic accumulation of fat in the liver, muscle, and possibly other tissues, may be responsible for its long-term adverse metabolic effects.

Our study has several weaknesses, however. First, it included a small number of subjects and, hence, may not have the statistical power required to detect small differences. To compensate for

this weakness, we however performed an 8-h metabolic monitoring with multiple measurements in very well controlled conditions. Second, it was an outpatient study, with no direct assessment of adherence to intervention. Every participant received every other week her/his estimated number of beverage cans needed, and adherence assessed from counting the empty cans returned at the following visit was highly satisfactory, however. Furthermore, we observed drastic decreases in 24-h urinary fructose excretion after intervention, supports the hypothesis that adherence was high. Third, we assessed the intake of solid foods and physical activity with methods known to have low sensitivity and, hence, cannot estimate how much of the SSBs' energy was compensated for by increased solid food consumption or decreased physical activity in the ASB group. Fourth, we have not included an intervention arm with water replacing SSBs and, hence, cannot assess whether artificial sweeteners exerted direct metabolic effects.

5. Conclusions

The present results indicate that a 12-week replacement of SSBs with ASBs failed to significantly change postprandial plasma triglyceride and uric acid concentrations in overweight subjects. This observation remained valid when the analysis was restricted to a subgroup of participants with hepatic steatosis. Although they may appear as unexpected, these results are consistent with several other published studies. The overall interpretation and practical implications of this study, and of the scientific literature on sucrose and health at large, may appear somewhat confusing. We believe that sucrose, given its high consumption in most of the world on one hand, and its hedonic properties on the other hand, is very likely to be an important contributor to the high prevalence of obesity. We also believe that SSBs, whose consumption is dependent on thirst and social occasions, rather than hunger, are particularly problematic. However, the hypothesis that fructose, per se, has adverse metabolic effects even when consumed as part of an energy-balanced diet may be somewhat exaggerated. Few intervention studies, having used large doses of fructose-containing sugars, actually support it. Our present results instead show that the postprandial metabolic profile was not markedly different when participants reduced their usual SSB consumption. Based on studies like this one, and on other reports showing no dramatic improvement of cardio-metabolic markers after SSBs reduction, one may have to reconsider the proposal that SSB reduction will be efficient to prevent or revert obesity unless associated with other drastic public health interventions. Public health interventions on SSBs, although entirely appropriate, should, therefore, not distract us from other, energy, fat, or salt-rich foods.

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Conflicts of Interest: L.T. received speaker's honoraria from Ferrero, Italy, the Gatorade Sport Science Institute, USA, and C3 collaborating for health, UK. The other authors have no conflict of interest to disclose.

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nutrients

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Review

Fructose and NAFLD: The Multifaceted Aspects of Fructose Metabolism

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Abstract: Among various factors, such as an unhealthy diet or a sedentarity lifestyle, excessive fructose consumption is known to favor nonalcoholic fatty liver disease (NAFLD), as fructose is both a substrate and an inducer of hepatic de novo lipogenesis. The present review presents some well-established mechanisms and new clues to better understand the pathophysiology of fructose-induced NAFLD. Beyond its lipogenic effect, fructose intake is also at the onset of hepatic inflammation and cellular stress, such as oxidative and endoplasmic stress, that are key factors contributing to the progression of simple steatosis to nonalcoholic steatohepatitis (NASH). Beyond its hepatic effects, this carbohydrate may exert direct and indirect effects at the peripheral level. Excessive fructose consumption is associated, for example, with the release by the liver of several key mediators leading to alterations in the communication between the liver and the gut, muscles, and adipose tissue and to disease aggravation. These multifaceted aspects of fructose properties are in part specific to fructose, but are also shared in part with sucrose and glucose present in energy–dense beverages and foods. All these aspects must be taken into account in the development of new therapeutic strategies and thereby to better prevent NAFLD.

Keywords: fructose; nonalcoholic fatty liver disease; liver; gut; muscle

1. Introduction

Nonalcoholic-fatty liver disease (NAFLD) represents a spectrum of disorders ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis, and liver cancer [1]. Its prevalence increases with that of type 2 diabetes, obesity, and metabolic syndrome [2] and is considered to be on average 20%–25%. Although several factors may contribute to NAFLD [3], fructose consumption is considered as a key player in the development of this disease [4,5], and it has repeatedly been reported to induce NAFLD in humans [6,7] and rodents [8,9]. A significant consumption of fructose leads to hepatic lipid accumulation and steatosis, steatosis being considered pathological when an abnormal accumulation of lipid droplets is observed in the cytoplasm of at least 5% of hepatocytes [4]. At this point, hepatic steatosis (HS) may be reversed through nutritional and physical exercise approaches [10,11]. Adversely, chronic consumption of fructose promotes several processes such as inflammation and cellular stress, which is responsible for the irreversibility of hepatic disorders and the progression of the disease [4]. The current review also provides new insights into the metabolic consequences of high fructose intake on peripheral tissues contributing to NAFLD progression.

2. Fructose and Hepatic Steatosis

An activation of the lipogenic program already occurs after a single load of fructose, leading to hepatic lipid accumulation [12–14]. As described below, this is the consequence of facilitated hepatic fructose metabolism for lipid synthesis and of the activation of signaling pathways whereby fructose promotes de novo lipogenesis (DNL).

2.1. Fructose as a Substrate of Hepatic de novo Lipogenesis

Fructose is subjected to rapid unregulated entry into the liver mainly via the glucose transporter 2 (GLUT2). At cell level, this carbohydrate is preferentially converted into fructose-1-phosphate (F1P) by fructokinase, which presents a high affinity for fructose, is not controlled by insulin, and is induced by fructose [15]. Thereafter, phosphotrioses produced from F1P through the action of aldolase B can be converted into glucose, lactate, and fatty acids [16]. While the lipogenic pathway is quantitatively minor in physiological situations, it becomes very active after an acute fructose load [12,17] as the flux of fructose carbons into lipogenic precursors increases, since the formation of F1P bypass the glycolysis regulatory site of phosphofructokinase1. Unregulated entry and metabolism of fructose into hepatocytes explain why, with high fructose diets, significant amounts of this carbohydrate continue to enter glycolysis and lead to excess acetyl-CoA production, relative to liver oxidative capacities, thus promoting DNL. High consumption of fructose also leads, by saturating the glycolytic pathway, to an accumulation of glycolysis intermediates which can be converted to glycerol-3-phosphate used in triglyceride (TG) synthesis.

2.2. Fructose as an Inducer of De Novo Lipogenesis

Chronic intake of fructose increases DNL by activating several key transcription factors [12] such as Sterol Response Element Binding Protein 1c (SREBP1c) and Carbohydrate-Responsive Element-Binding Protein (ChREBP) [17,18]. As a consequence, their key target enzymes regulating lipid synthesis, such as Fatty Acid Synthase (FASN) and Acetyl-CoA Carboxylase (ACC), also increase as shown for example in rodents submitted to a 60% high fructose diet for eight weeks [18] or to a western diet where fructose is provided as a 30%-fructose containing beverage for eight weeks [19].

Thus, as fructose is both substrate and activator of DNL, it appears as the most potent lipogenic carbohydrate contributing to the development of liver steatosis.

3. Fructose and Disease Progression

Fructose by itself or via increased DNL may promote oxidative stress, in part via mitochondrial dysfunction and endoplasmic reticulum (ER) stress, both contributing to the development of an inflammatory process and the progression of simple steatosis to NASH.

3.1. Fructose and Oxidative Stress

Fructose induces oxidative stress *via* several mechanisms. First, because fructose is structurally different from glucose, it can promote more hepatocellular damage. Acute fructose load induces protein fructosylation. This reaction is non-enzymatic and is seven times faster than glycation by glucose. In addition, fructose generates 100 times more reactive oxygen species (ROS) than glucose [4]. Compared with glucose, prolonged fructose feeding in mice led to a higher hepatic accumulation of carboxymethylysine, a glycation product that, for example, can interact with SREBP-cleavage activating protein to induce sustained SREBP1c activation [20].

Second, fructose phosphorylation in the liver consumes adenosine triphosphate (ATP): As phosphorylation by fructokinase is fast and the cleavage reaction by aldolase B relatively slow, an excess of fructose could cause hepatic phosphate deficiency, leading to AMP accumulation with resulting increased uric acid synthesis [7,21]. Uric acid in turn stimulates the production of ROS [22]

via the activation of Transforming Growth Factor β and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 ([23].

Third, hepatic metabolism of fructose generates other molecules such as methylglyoxal (MG), a potent glycating agent leading to cellular stress and altered insulin signaling [24]. In condition of standard feeding, MG formation rate represents 0.1%–0.4% of glycolytic flux [25] but accelerated glycolytic flux with fructose increases MG formation.

Last, mitochondrial dysfunction may also be induced by the lipotoxicity related to the fructose-induced perturbation of hepatic lipid metabolism [8]. Mechanisms involved may be (i) a decrease in lipid degradation due to a lower expression of Peroxisome Proliferator-Activated Receptor α (PPARα) that regulates genes involved in β-oxidation such as Carnitine Palmitoyl Transferase 1 (CPT1) [18], a lower expression of the peroxisomal proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) (a mitochondrial-biogenic protein) [26]; and (ii) a decrease in lipid clearance due to a lower expression of Microsomal Triglyceride Transfer Protein (MTP) [18] involved in Very Low Density Lipoprotein (VLDL) production. However, the exact mechanisms remain debated as, in some studies, an enhancement of beta-oxidation and VLDL-clearance after fructose consumption has been reported, suggesting that hepatic lipid accumulation mainly results from uncontrolled DNL [27]. As a result, the disequilibrium between DNL and VLDL release may promote alterations of the respiratory chain and to the uncoupling of oxidative phosphorylation with excess ROS production [28,29]. Mitochondria and ER being associated through mitochondria-associated ER-membrane plays a key role in calcium signaling and lipids transfer, ROS overproduction by mitochondria contributes to ER stress, and hepatic inflammation, two processes addressed in the following sections.

3.2. Fructose and Endoplasmic Reticulum Stress

Studies pointed to ER stress as a mechanism favoring HS progression to NASH [30]. Chronic fructose consumption leads to a higher solicitation of the ER via the stimulation of lipid metabolism and of VLDL-TG production. ER membrane proteins may be fructosylated, or lipids may accumulate into ER membrane leading to ER stress and the unfolded protein response (UPR). Although UPR activation first allows the restoration of ER homeostasis, during sustained fructose exposure ER stress becomes chronic leading, to inflammation, oxidative stress, and apoptosis [31,32]. This also contributes to the progression of hepatic steatosis and of insulin resistance [33]: ER stress further interferes with lipid metabolism in the liver by activating DNL, via the protein kinase activated by dsRNA (PKR)-related Endoplasmic Reticulum Kinase (PERK)/eukaryotic translation Initiation Factor 2α (eIF2α)/Activating Transcription Factor 4 (ATF4) pathway and by limiting the formation and secretion of VLDL, via Inositol Requiring Enzyme 1 (IRE1) pathway. ER stress also acts indirectly on the accumulation of TG in the liver by inducing hepatic and adipose tissue insulin resistance. Furthermore, ER stress promotes the activation of transcription factors Janus kinase (JNK), Nuclear Factor κB (NFκB), ChREBP, SREBP, and CCAAT/enhancer-binding protein homologous protein (CHOP), which are involved in inflammatory processes and cell death and play an important role in the progression of NAFLD [33].

3.3. Fructose and Inflammation

The contribution of fructose diet to the inflammatory process is well established [34]. The specific role of hepatic fructose metabolism in liver inflammation is suggested by the protective effect of fructokinase knockout against high-fat high-sucrose-induced steatohepatitis [3]. Ectopic liver fat accumulation increases hepatocytes vulnerability to cellular stress, therefore initiating an inflammatory process [35]. In parallel, cellular stress can be exacerbated by toll-like receptor 4 (TLR4) activation-induced inflammation in Kupffer cells since fructose has been shown to promote the synthesis of saturated fatty acids such as palmitate, which are able to activate TLR4 receptors in the liver [36]. The activation of the TLR4/inducible nitric oxide synthase (iNOS)/NFκB pathway induces oxidative stress in hepatocytes via the production of pro-inflammatory cytokines, such as

tumor necrosis factor (TNF) α by Kupffer cells. These phenomena are reinforced by the lipid-induced increase in the proportion of "conventional" pro-inflammatory M1 macrophages relative to "alternate" anti-inflammatory M2 macrophages [37]. In a study in 427 patients with NAFLD, fructose consumption has been shown to be associated with increased hepatic fibrosis, in keeping with a fructose-induced increase in hepatic inflammation and ER stress [7]. Last, fructose also modulates liver inflammation by inducing dysbiosis as discussed in detail below.

The superimposition of ER stress and inflammation may lead to the production of various mediators such as cytokines, hepatokines, carbohydrates, and lipid derivatives collectively known as DAMPs (damage associated molecular pattern) that signal at the whole-body level and contribute to alterations in whole body metabolism. Many studies, both in animal models [18,19] and more recently in patients with NASH, show that abnormal hepatokines production also plays a key role in the pathogenesis of NASH [38,39]. Hepatokines such as Fetuin A, Fibroblast growth factor 21 (FGF-21), Leucocyte cell-derived chemotaxin 2 (LECT2), and Angiopoietin-like protein (ANGPTL) released by the steatotic liver may contribute to peripheral organ dysfunction [40,41].

4. Fructose and Interorgan Cross-Talks

The following sections review some of the mechanisms whereby fructose directly or indirectly, through the release of lipids, hepatokines, and uric acid into the blood, leads to alterations in gut, muscle, and adipose tissue functions (Figure 1).

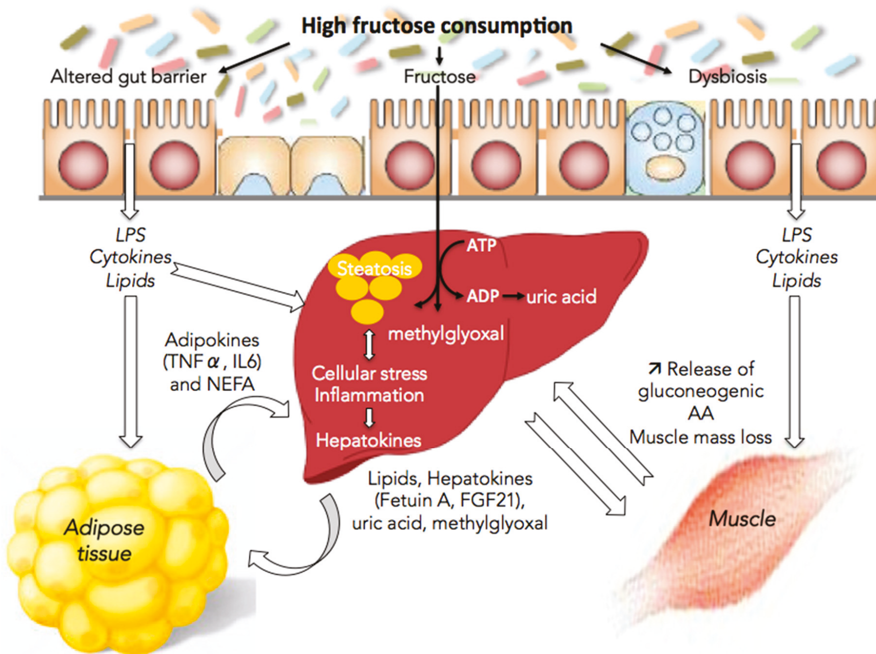

Figure 1. Fructose and nonalcoholic fatty liver disease (NAFLD): the multifaceted aspects of fructose metabolism. Excessive fructose consumption is associated with hepatic steatosis, cellular stress and inflammation. This is responsible for the release by the liver of lipids, methyglyoxal, uric acid, and hepatokines leading to alterations in the communication between the liver and the gut, muscles, and adipose tissue and to disease aggravation. LPS, lipopolysaccharides; TNFα, tumor necrosis factor α; IL6, interleukin-6; NEFA, non-esterified fatty acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; FGF21, fibroblast growth factor 21; AA, amino acids.

4.1. Fructose and the Gut/Liver Axis

The progression of HS to NASH is also influenced by gut function and the possible translocation of bacterial compounds due to a compromised intestinal barrier [42,43]. First, insulin resistance by itself is already associated with alterations in gut permeability [44]. In parallel, patients with NAFLD present a dysbiosis characterized by an increase in *Clostridium coccoides* and a decrease in Bacteroides/Prevotella [45]. Fructose-induced NAFLD is also associated with changes in microbiota composition [46] that alters gut permeability by reducing expression of tight junction proteins [19,47]. As a consequence of this alteration in gut barrier function and of the dysbiosis, NASH, and cirrhotic patients present an increase in endotoxin translocation [45,48]. The ensuing activation of TLR4 in Kupffer cells and infiltrated monocytes worsen innate and adaptive immune responses. Liver exposition to endotoxins such as lipopolysaccharides (LPS) may induce a chronic inflammation associated with a recruitment of neutrophils that release ROS, proteases, lipocalin-2, and enzymes leading to an aggravation of liver injuries [8,49]. LPS and oxidative stress also activate stellar cells leading to fibrosis. Thereafter, cytokines activate several signaling pathways such as the pro-apoptotic pathways [50]. Gut barrier alteration also promotes hepatic macrophages polarization to M1 phenotype further favoring inflammatory liver injury [51].

Last, enterocytes also metabolize a small part of fructose into lactate, glucose, and also TG. Theytaz et al. [21] demonstrate an increase in both ^{13}C-palmitate chylomicron and ^{13}C-palmitate VLDL-TG concentrations after a ^{13}C-fructose load in non-obese, young human subjects. This may contribute to the alteration of metabolism and ultimately of liver function [21,52].

Thus, it seems important to consider the gut-liver axis in the management of NASH or of other NAFLD stages.

4.2. Fructose and Adipose Tissue/Liver Axis

Visceral fat mass increases with fructose diet in humans [53] as well as in experimental models [18,54]. This suggests either a direct fructose metabolism in visceral adipocytes, which may be exposed to higher fructose concentrations than subcutaneous adipocytes due to anastomosis between portal hepatic and systemic splanchnic circulation, or an indirect effect through an accumulation of lipid originating from the liver. Vràna et al. [55] showed an inhibition of DNL in adipose tissue in fructose-fed rats. The increase in fructose-derived MG production by the liver may play a role as Masterjohn et al. [56] showed that fructose-fed rats display an accumulation of MG in epididymal adipose tissue. MG alters insulin signaling pathway in visceral adipose tissue in vivo [57]. In vitro, fructose increases adipogenesis and, conversely, the inhibition of fructose transport in mice is associated with reduced epididymal adipose tissue [58]. Together these data underline fructose influence on visceral adipose tissue but data in human are missing.

Owing to this adipogenic effects, adipokines and cytokines profile would also be changed by fructose diet. The consequences of this increased visceral adiposity are elevated circulating free fatty acids and proinflammatory mediators. Due to the anatomic proximity and the portal circulation, this will clearly alter liver function but also other the function of peripheral organs leading to an aggravation of the metabolic disorders [34].

4.3. Fructose and Muscle/Liver Axis

A high-fructose diet is associated with modifications in muscle function [59] in humans [26] and in rodents [60]. Mechanisms involved in diet-induced sarcopenia may be (i) a decrease in mechanistic target of rapamycine complex (mTORC) 1 activity, and thereafter in protein synthesis [61]; and (ii) inflammation [62]. Recent studies in fructose-fed rats have shown an association between NAFLD and sarcopenia [63]. This is a key factor involved in disease progression to NASH as the muscle heavily contributes to energy homeostasis [64]. Gatineau et al. [65] recently showed in aged rats that

sucrose-fed animals lost significantly more lean body mass and retained more fat mass than starch-fed rats and presented lower meal-induced stimulation of muscle protein synthesis.

Disorders of nitrogen homeostasis in situations of stimulated DNL may be an early event following excessive fructose consumption [12,51] as excess fructose may alter liver-muscle axis via its metabolism or via DNL-associated RE stress leading to increased production by the liver of catabolic effectors. First, the increase in lipid flux observed with fructose-enriched diet contributes to alter muscle insulin sensitivity [59,66,67]. Second, as previously described, excess fructose may lead to a saturation of its normal metabolism with adverse consequences in terms of increased hepatic release of MG or uric acid. In vitro studies show that MG inhibits insulin signaling in muscle [68]. High fructose diet under hypercaloric feeding conditions has been shown to induce hyperuricemia that contributes to metabolic disorders [69,70]. Uric acid inhibits muscle insulin signaling and induces insulin resistance in mice [71] as well as in severely obese subjects [72]. Third, hepatic ER is associated with enhanced production of pro-inflammatory cytokines and hepatokines suspected to be involved in alterations in energy homeostasis and insulin-resistance. ER stress markedly stimulates liver production of Fetuin A [73] and of insulin-like growth factor binding protein 1 (IGFBP1) [74]. Fetuin A is an endogenous inhibitor of the insulin receptor tyrosine kinase in muscle [75], while IGFBP1 is a modulator of insulin-like growth factor 1 (IGF-1) action associated with hyperinsulinemia and glucose intolerance [76]. ER stress modulates fibroblast growth factor 21 (FGF21) expression in the liver [77]. FGF21 is a mediator mainly produced by the liver that contributes to the regulation of peripheral energy metabolism and insulin sensitivity [78]. It is now recognized as a key player in the adaptive response to starvation and feeding [79]. Last, fructose consumption leads to decreased liver production of anabolic factors such as insulin-like growth factor (IGF)1 [64].

Another factor contributing to these alterations of protein metabolism is a reorientation of AA fluxes as suggested by NAFLD-associated changes in plasma amino acids (AAs) profile [18]. In hypertriglyceridemic patients, fructose increased plasma arterial AA concentrations but also their splanchnic extraction [80]. These interorgan AA fluxes probably correspond to a reorientation of AAs towards the liver in order to enable the synthesis of inflammatory proteins and the elevated gluconeogenesis. In situations of fructose overfeeding, energy metabolism would be oriented towards an increase in gluconeogenesis and DNL and a decrease in lipid catabolism. Conversely, a regulatory role of AA availability on liver DNL has been shown in experimental and human studies as increased AA availability prevents hepatic lipid accumulation via (i) a decrease in DNL through decreased gene expression of ChREBP, SREBP-1c and Fas (ii) an increase in ß-oxidation through increased gene expression of PPARα; and (iii) an enhance in VLDL production through increased gene expression of MTP [18]. AA supplementation has also been shown to decrease gene expression of TLR4 and interleukin-6 (IL6) in liver and to prevent the loss in lean body mass in fructose-fed rats [18]. The basis for this interaction between DNL, AA availability, and protein homeostasis needs to be confirmed in humans. Interestingly, in healthy volunteers, essential AA supplementation decreased fructose-induced intrahepatic lipid accumulation [21].

5. Specific or Indirect Effect of Fructose

The above-mentioned peripheral manifestations associated with fructose feeding and several short-term studies, using ^{13}C-fructose as metabolic tracer, suggest a specific effect of fructose. Although it has long been taught that fructose is mainly metabolized in the liver [16], a small part of this carbohydrate may bypass liver extraction and be metabolized in extrahepatic cells since various cells, including neurons, express fructose transporter GLUT5 and enzymes involved in its metabolism [20,56,81]. However, data in human are missing.

Moreover, the exact contribution of fructose intake is frequently blurred by the associated imbalance in energy homeostasis. Fructose is often consumed in diets also rich in glucose and lipids. Although this carbohydrate is more harmful than glucose as it is more lipogenic and its metabolism differs from that of glucose [8,45], its effects are amplified when it is associated with glucose [82].

Indeed, fructose effects are more severe when consumed in the form of disaccharides (i.e., sucrose composed of equal parts of fructose and glucose) or associated with other macromolecules such as lipids [19,83].

6. Fructose and NAFLD Management

Lifestyle changes, including physical activity and balanced diets, are the initial treatment of steatosis, especially when they enable to lose weight. In NASH patients, a 3% to 7% weight loss is associated with decreased hepatic steatosis [84]:

Exercise prevents fructose-induced hypertriglyceridemia in healthy subjects and promotes a decrease in hepatic TG content [85]. In patients with NASH, markers of disease severity are decreased after 200 min per week of moderate intensity physical activity for 48 weeks, associated with a balanced diet [86].

Apart from limiting caloric intake, these patients should avoid a diet rich in saturated fatty acids, sucrose, and alcohol [3,87]. For example, the Mediterranean diet rich in mono-unsaturated fatty acids may be effective [88]. It has been shown to reduce liver steatosis and improve insulin sensitivity in patients with NAFLD without diabetes [89]. Diets enriched with omega-3 polyunsaturated fatty acids may also reduce steatosis [90].

A more dramatic strategy to induce weight loss is bariatric surgery. It is an effective procedure to improve insulin resistance and glucose metabolism primarily by reducing calorie intake, thereby reducing body weight and liver steatosis [91].

Other possible alternatives are pro- and prebiotics, which are of growing interest in the management of these patients because of their effect on gut microbiota and/or gut barrier function. For example, *Lactobacillus rhamnosus* GG protects against the development of fructose-induced NAFLD via the preservation of gut microbiota thus restoring the intestinal barrier via increased expression of Claudine-1 and Occludine tight junction proteins [47]. Last, there is an increasing interest in natural products and plant extracts that could be effective on some aspects on fructose-induced NAFLD [92,93]. However, their clinical effectiveness remain to be evaluated.

In NAFLD patients with metabolic syndrome or type 2 diabetes, lipid-lowering therapy or insulin-sensitizing agents have been proposed. Statins, fenofibrate, and ezetimibe treatments result in only modest improvement to liver damage in NASH patients. The effects of the insulin-sensitizer metformin are debated: while Bergheim showed that metformin protects mice against fructose-induced NAFLD [94], metformin does not improve the histological alterations observed in people with NASH [95]. Concerning thiazolidinediones, their side effects preclude their use in NAFLD [95].

7. Conclusions

Based on its specific splanchnic (predominantly hepatic) metabolism, on its lipogenic potential, and on its high consumption in modern diets, fructose appears as one major factor not only of the initiation of hepatic steatosis, but also of its progression to NASH and more severe stages of the disease. Understanding its metabolism may provide novel opportunities for therapeutic intervention.

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Abbreviations

AA: amino acid; ADP, Adenosine Diphosphate; ACC: Acetyl CoA Carboxylase; ANGPTL: Angiopoietin-like protein; ATF4: Activating Transcription Factor 4; ATP: Adenosine Triphosphate; CHOP: CCAAT-enhancer-binding protein homologous protein; ChREBP: Carbohydrate-Responsive Element-Binding Protein; CPT1: Carnitine Palmitoyl Transferase 1; DAMP: damage associated molecular pattern; DNL: de novo lipogenesis; eIF2α: eukaryotic translation Initiation Factor 2α; ER: endoplasmic reticulum; F1P: fructose-1-phosphate; FASN: Fatty Acid Synthase; FGF-21: Fibroblast Growth Factor 21; GLUT: glucose transporter; HS : hepatic steatosis; IGF: insulin-like growth factor; IGFBP: IGF binding protein1; iNOS: inducible nitric oxide synthase; IL6: Interleukin-6; IRE1: Inositol Requiring Enzyme 1; JNK: Janus kinase; LECT2: Leucocyte cell-derived chemotaxin 2; MG:

methylglyoxal; mTORC: mechanistic target of rapamycine complex complex; MTP: microsomal Triglyceride transfer Protein; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; NAFLD: nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; NEFA, Non-Esterified Fatty Acid; NFκB: Nuclear Factor κB; PERK: PKR-related Endoplasmic Reticulum Kinase; PKR: Protein Kinase Activated by dsRNA; PPARα: Peroxisome Proliferator-Activated Receptor α; ROS: reactive oxygen species; SREBP1c: Sterol Response Element Binding Protein 1c; TG: triglycerides; TLR4: toll-like receptor 4; TNF: tumor necrosis factor; UPR: unfolded protein response; VLDL: Very Low Density Lipoprotein.

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Article

The Addition of Liquid Fructose to a Western-Type Diet in LDL-R$^{-/-}$ Mice Induces Liver Inflammation and Fibrogenesis Markers without Disrupting Insulin Receptor Signalling after an Insulin Challenge

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Abstract: A high consumption of fat and simple sugars, especially fructose, has been related to the development of insulin resistance, but the mechanisms involved in the effects of these nutrients are not fully understood. This study investigates the effects of a Western-type diet and liquid fructose supplementation, alone and combined, on insulin signalling and inflammation in low-density lipoprotein (LDL) receptor-deficient mice (LDL-R$^{-/-}$). LDL-R$^{-/-}$ mice were fed chow or Western diet ±15% fructose solution for 12 weeks. Plasma glucose and insulin, and the expression of genes related to inflammation in the liver and visceral white adipose tissue (vWAT), were analysed. V-akt murine thymoma viral oncogene homolog-2 (Akt) activation was measured in the liver of the mice after a single injection of saline or insulin. None of the dietary interventions caused inflammation in vWAT, whereas the Western diet induced hepatic inflammation, which was further enhanced by liquid fructose, leading also to a significant increase in fibrogenesis markers. However, there was no change in plasma glucose or insulin, or insulin-induced Akt phosphorylation. In conclusion, hepatic inflammation and fibrogenesis markers induced by a Western diet supplemented with liquid fructose in LDL-R$^{-/-}$ mice are not associated with a significant impairment of hepatic insulin signalling.

Keywords: simple sugars; high-fat diet; adipose tissue; inflammasome; metabolism

1. Introduction

Unhealthy nutritional habits such as the excessive consumption of fat and refined sugars, together with a sedentary way of life, are linked to metabolic alterations including non-alcoholic fatty liver disease (NAFLD)—an array of conditions ranging from simple steatosis to steatohepatitis. NAFLD is strongly associated with insulin resistance (IR). However, the role of hepatic lipid accumulation and inflammation in the alterations of insulin signalling which will lead to IR is not fully understood. For example, although in animal models hepatic steatosis has been generally linked to the development of IR, this association is not so straightforward in the studies in which adiposity and body weight are not affected [1]. On the other hand, inflammation has been proposed as the link between NAFLD and IR, as several murine models in which components of the nuclear factor κ B (NFκB) inflammatory

pathway are constitutively activated or deleted show that increased hepatic inflammation correlates with an increase in IR [2] and, conversely, a reduction in hepatic inflammation improves hepatic insulin sensitivity [3–5]. However, the evidence is not so clear, as other murine models show a dissociation between inflammation in the liver and IR [6–11]. For example, mice with a gain-of-function mutation in tumour necrosis factor receptor 1 display hepatic inflammation, but systemic and hepatic IR are not affected and adipose tissue inflammation is not observed [10].

The food constituents that have been linked to the development of metabolic disturbances such as NAFLD and IR include simple sugars, especially fructose. Our research group has shown that liquid fructose supplementation in female rats fed a standard chow diet (two weeks to two months) induces glucose intolerance and IR [12–15]. However, unhealthy human dietary patterns frequently include not only excessive added sugars, but also excessive fat intake, particularly saturated fats [16]. For this reason, in a recent publication we explored the effects of liquid fructose supplementation to C57BL/6N mice in two different solid diets, a standard rodent chow and a Western-type rodent chow rich in saturated fat, refined carbohydrates and cholesterol [17]. Our results showed that in this model liquid fructose supplementation increased liver triglyceride and cholesterol levels and diminished whole-body insulin sensitivity only when it was combined with a Western-type diet; however, these effects were not associated with a clear inflammatory reaction in the liver [17]. We also studied the effects of these diets on the development of aortic atherosclerotic lesions in low-density lipoprotein (LDL) receptor-deficient (LDL-$R^{-/-}$) mice [18]. As it has been shown that the LDL-$R^{-/-}$ mouse is a good model for studying the progression from steatosis to inflammation in the liver [19], it can be helpful to better understand the relationship between hepatic lipid accumulation, inflammation and IR. Here, we investigate the effects on insulin signalling and inflammation of liquid fructose supplementation and a Western-type diet, alone and combined, using samples from our previous study conducted on LDL-$R^{-/-}$ mice [18].

2. Materials and Methods

2.1. Animals and Diets

The animal experimental protocols were described in our previous publication [18]. Briefly, male LDL-$R^{-/-}$ mice were randomly assigned to four groups (n = 10/group): 1. Control group (CT), which was fed a standard rodent diet (2018 Teklad Global 18% Protein, Envigo, Barcelona, Spain) without any supplementary sugar; 2. Fructose-supplemented group (F), which received the same standard diet supplemented with 15% weight/volume fructose in drinking water; 3. Western group (W), which was fed a Western-type diet (D12079B Open Source Diets, Research Diets Inc., New Brunswick, NJ, USA) without any supplementary sugar; 4. Western plus fructose group (WF), which received the Western-type diet supplemented with 15% weight/volume fructose in drinking water. The composition of control and Western-type diets was as detailed previously [18]. After 12 weeks, the animals were fasted for 2 h and blood was obtained from the tail vein to determine glucose concentrations. Six animals from each group were then euthanized under intraperitoneal ketamine (100 mg/kg)/xylazine (10 mg/Kg) anaesthesia. Blood samples were obtained by intracardiac puncture, collected in microtubes (Sarstedt AG & Co., Nümbrecht, Germany), and plasma was obtained by centrifugation and stored at −80 °C until use. Liver and visceral white adipose tissue (vWAT) was excised, immediately frozen in liquid N_2 and stored at −80 °C until use. The remaining animals (n = 4/group) were used to study insulin signalling in the liver. To this end, mice were fasted for 12 h, anaesthetised with ketamine:xylazine, as described above, and intraperitoneally injected with 0.15 units of insulin/g body weight (Humulina® Regular, Lilly, Madrid, Spain). Fifteen minutes later the livers were obtained, frozen in liquid N_2 and stored at −80 °C.

All procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee, as stated in Law 5/1995, of 21 July, from the Catalan government

(approval code 6078). These guidelines adhere to Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.2. Blood and Plasma Analysis

Blood glucose levels were measured using an Accutrend® Plus system glucometer (Cobas, Roche Farma, Barcelona, Spain). Insulin concentration in plasma was determined using an ELISA kit (Millipore, Billerica, MA, USA). Whole-body insulin sensitivity was estimated by calculating the Insulin Sensitivity Index (ISI) [20], and the HOMA-IR [21]. Alanine aminotransferase (ALT) activity in plasma samples was determined using an ALT/GPT Spinreact kit (Spinreact, Girona, Spain), based on the spectrophotometric measurement of the rate of reduction in NADH levels. To determine lipopolysaccharide-binding protein (LBP) levels, we used an ELISA kit from Hycult Biotech (Uden, The Netherlands).

2.3. Histological Studies

Paraffin-embedded liver sections were stained with hematoxylin and eosin to assess necrosis and with Masson's trichrome acid to determine the degree of fibrosis. Images were acquired with an Olympus BX43 microscope (Olympus Iberia, Barcelona, Spain) and a pathologist blinded to the treatment groups performed the histological analysis at BioBanc (Banc de tumors-IDIBAPS, Barcelona, Spain). Necrosis was scored as 0 (absent), 1 (<1%), 2 (<5%), 3 (<10%) or 4 ($\geq$10%).

Fibrosis was categorised as 0 (no fibrosis), 1 (portal or sinusoidal fibrosis without septa), 2 (portal or sinusoidal fibrosis with rare septa), 3 (abundant septa without cirrhosis) or 4 (cirrhosis).

2.4. RNA Preparation and Analysis

Total RNA was isolated by using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Specific mRNAs were assessed by real-time reverse transcription polymerase chain reaction (RT-PCR), using SYBR Green PCR Master Mix, specific primers and the Applied Biosystems StepOnePlus sequence detection system (Applied Biosystems, Foster City, CA, USA). TATA box-binding protein (TBP) was used as an internal control. Primer sequences and PCR product length are listed in Supplemental Table S1.

2.5. Western Blot Analysis

Total protein extracts from liver were obtained by the Helenius method [22], and protein concentrations were determined by the Bradford method [23]. Thirty micrograms of protein extracts were subjected to SDS-polyacrylamide gel electrophoresis. The protein fractions were then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Billerica, MA, USA), blocked for 1 h at room temperature with 5% non-fat milk solution in 0.1% Tween-20-Tris-Buffered Saline (TBST), and incubated as described previously [24]. Detection was performed using the ECL chemiluminescence kit for HRP (Amersham GE Healthcare Europe GmbH, Barcelona, Spain). To confirm the uniformity of protein loading, blots were incubated with β-tubulin antibody (Sigma-Aldrich, St. Louis, MO, USA) as a control. Primary antibodies for phosphorylated and total Akt and IκBα were supplied by Cell Signaling Technology (Danvers, MA, USA) and those for IRS-2 and phosphorylated and total IRβ were obtained from Santa Cruz Biotechnologies (Dallas, TX, USA). The antibody against phosphorylated IRS-2 was from Abcam (Cambridge, UK).

2.6. Statistical Analysis

The results are expressed as the mean of n values $\pm$ SEM. Plasma samples were assayed in duplicate. Significant differences between the values from the CT, F and W groups were established by one-way ANOVA and Šidák's post-hoc test for selected comparisons; significant differences between the values from the W and WF groups were established by the unpaired t-test (GraphPad Software

V6, La Jolla, CA, USA). When variance was not homogeneous, a non-parametric test was performed. The correlations between liver total cholesterol content and inflammation-related parameters were examined by two-tailed Pearson or Spearman Correlation analyses for data which follow a Gaussian distribution or not, respectively. The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Western Diet ± Liquid Fructose Supplementation Does Not Trigger Visceral White Adipose Tissue Inflammation in LDL-R$^{-/-}$ Mice

Zoometric parameters and liver lipid content in the animals used in the present study were previously reported [18], and are shown in Supplemental Table S2. Mice fed a Western-type diet (W group) showed an increase in body weight at the end of the study compared to controls (C), and the addition of liquid fructose (WF group) further increased final body weight. Importantly, liver and vWAT weights were only significantly increased in the WF group compared to the W group, despite the total amount of calories ingested by the mice from these groups were almost identical.

Using vWAT samples of LDL-R$^{-/-}$ mice from the different dietary groups, we examined the expression of several pro-inflammatory molecules (Figure 1). The mRNA expression of toll-like receptor 4 (*tlr4*), which plays an essential role in the induction of pro-inflammatory cytokines in adipocytes, showed no differences among the groups. Similarly, the mRNA levels of the adaptor protein myeloid differentiation factor-88 (*myd88*) remained unaffected. In accordance with the lack of activation of the TLR4 pathway, the expression of the inflammatory cytokine tumour necrosis factor α (*tnf*α) was not significantly altered in vWAT in any of the dietary groups. C-C chemokine receptor type 2 (*ccr2*), which is involved in monocyte chemotaxis, showed a significant increase in the WF group compared to the W group ($p < 0.01$). However, the mRNA levels of monocyte chemoattractant protein-1 (*mcp-1*) and macrophage marker *f4/80* tended to increase, but not significantly, in these groups.

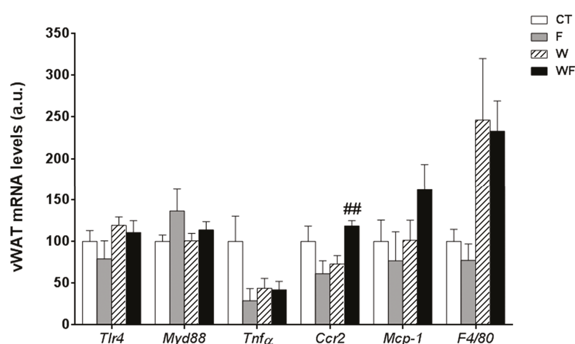

Figure 1. Bar plots showing the relative levels of specific mRNAs of pro-inflammatory molecules from Control (CT, mice fed standard solid-chow), Fructose (F, mice fed standard solid-chow plus a 15% fructose solution ad libitum), Western (W, mice fed Western solid-chow), and Western + Fructose (WF, mice fed Western solid-chow plus a 15% fructose solution ad libitum), represented as mean (a.u., arbitrary units) ± SEM of five different visceral adipose tissue samples. ## $p < 0.01$ vs. W values.

3.2. A Western-Type Diet Induces Hepatic Inflammation and Liquid Fructose Supplementation Enhances This Effect

In contrast to the lack of a clear inflammatory response in vWAT, the expression of most of the inflammatory markers determined in the liver was significantly increased by the Western-type diet alone and combined with liquid fructose supplementation (Figure 2). Thus, the expression of *tlr4* was induced by the Western diet (1.9-fold increase in W vs. CT group, $p < 0.01$), and supplementation

with liquid fructose in this diet further enhanced the effect (2.3-fold increase in WF vs. W group, $p < 0.01$), although *myd88* mRNA levels remained unaffected. In accordance with *tlr4* induction, the mRNA level of the pro-inflammatory cytokine *tnfα* was increased in W compared to CT (2.5-fold, $p < 0.05$), and further induced after the Western diet was supplemented with fructose (3.3-fold increase in WF vs. W group, $p < 0.01$). Regarding the expression of genes related to monocyte recruitment and macrophage accumulation, our results showed no significant modification in the expression of *ccr2*, and a marked increase in the mRNA of *mcp-1* only in the WF group compared to W (8.1-fold, $p < 0.01$), whereas the expression of the macrophage marker *f4/80* was increased by the Western diet and further increased by fructose supplementation in this diet (1.4-fold, $p < 0.05$ W vs. CT and WF vs. W).

Given the inflammatory response observed in the liver, we assessed the involvement of the inflammasome in this process. To this end, the mRNA levels of several components of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome were examined. As shown in Figure 3A, the mRNA expression of *nlrp3* and *caspase-1* were significantly higher in the WF group compared to the W group (2.5-fold, $p < 0.01$ and 1.8-fold, $p < 0.001$, respectively). Similarly, the mRNA expression of the adaptor molecule *asc* (apoptosis-associated speck-like protein containing a caspase-recruitment domain) was increased by the Western-type diet (1.3-fold, $p < 0.05$) and further enhanced by the addition of fructose to this diet (1.5-fold increase in WF vs. W group, $p < 0.01$).

One of the pathways that initiates inflammasome activation is mediated by microbial signals, such as lipopolysaccharide (LPS), which upregulate NLRP3 expression through nuclear factor κB (NFκB) [25]. Plasma level of LPS-binding protein (LBP), a biomarker of circulating LPS, was unaffected by fructose supplementation, but increased in the W group compared to the CT group (3-fold, $p < 0.05$) (Figure 3B). To determine NFκB activity, we measured the amount of phosphorylated inhibitor of κB (p-IκB), as the classical pathway of NFκB activation involves IκB phosphorylation and degradation, which favors NF-κB nuclear translocation and transcriptional activity. Our results show that p-IkB levels were increased significantly in the WF group compared to W (Figure 3C).

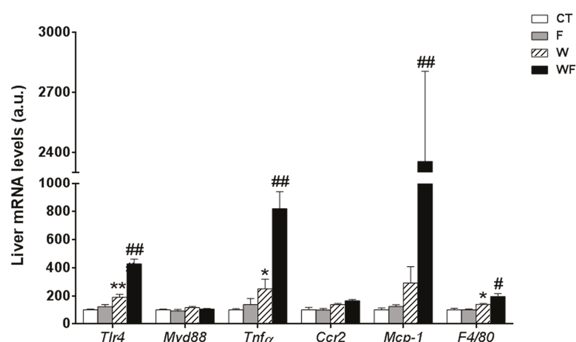

Figure 2. Bar plots showing the relative levels of specific mRNAs of pro-inflammatory molecules from Control (CT, mice fed standard solid-chow), Fructose (F, mice fed standard solid-chow plus a 15% fructose solution ad libitum), Western (W, mice fed Western solid-chow), and Western+Fructose (WF, mice fed Western solid-chow plus a 15% fructose solution ad libitum), represented as mean (a.u., arbitrary units) ± SEM of five different liver samples. * $p < 0.05$, ** $p < 0.01$ vs. CT values; # $p < 0.05$, ## $p < 0.01$ vs. W values.

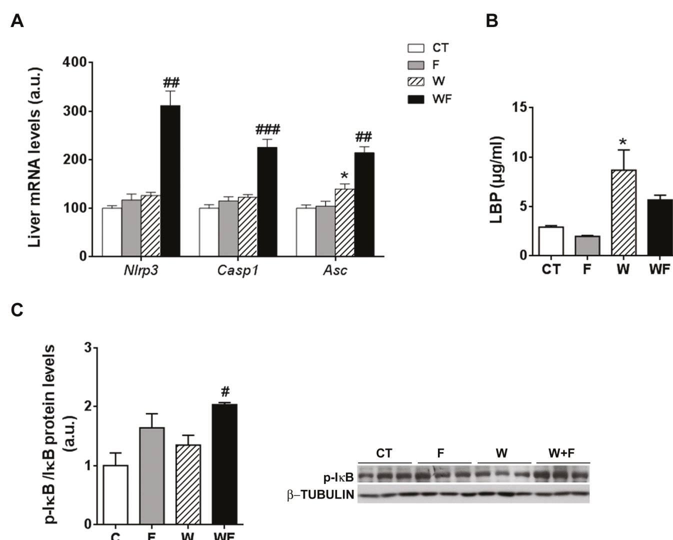

Figure 3. (**A**) Bar plots showing the relative levels of specific mRNAs of the NLRP3 inflammasome complex from Control (CT, mice fed standard solid-chow), Fructose (F, mice fed standard solid-chow plus a 15% fructose solution ad libitum), Western (W, mice fed Western solid-chow), and Western + Fructose (WF, mice fed Western solid-chow plus a 15% fructose solution ad libitum), represented as mean (a.u., arbitrary units) ± SEM of five different liver samples; (**B**) Plasma level of lipopolysaccharide (LPS)-binding protein represented as mean (μg/mL) ± SEM of five to six different plasma samples obtained from CT, F, W and WF groups; (**C**) Western-blot of phosphorylated and total IκBα in liver samples obtained from CT, F, W and WF groups. Representative bands corresponding to three different mice in each group are shown; bar plots show the level of the phosphorylated protein expressed as the mean (a.u., arbitrary units) ± SEM of the values obtained from three to four animals. * $p < 0.05$ vs. CT values; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. W values.

3.3. Addition of a Liquid Fructose Supplement to a Western-Type Diet Increases Fibrogenesis Markers in the Liver of LDL-R$^{-/-}$ Mice

Plasma ALT activity, which is commonly used to monitor liver injury, showed a marked increase in the WF group compared to the W group (6.8-fold, $p < 0.01$, Figure 4A), although hematoxylin and eosin staining did not reveal apparent necrosis in any of the groups (Figure 4B,C).

Then, we measured the expression of factors related to fibrogenesis key events such as stellate cell activation and matrix deposition. The hepatic mRNA levels of transforming growth factor β (*tgf-β*) and tissue inhibitor of metalloproteinase 1 (*timp-1*) were markedly and significantly increased only in the WF group, compared to the W group (Figure 4D,E). Consistent with this induction, hepatic collagen α1 (*collα1*) mRNA levels were markedly increased only in the WF mice (Figure 4F). We then assessed the degree of hepatic fibrosis by examining the Masson's trichrome-stained liver sections. As shown in Figure 4G,H, none of the diets caused overt liver fibrosis; the semi-quantitative analysis of fibrosis scores indicated that only in the fructose-supplemented groups there were 50%–70% of mice with score 1, and in the WF group there was one animal which reached grade 2.

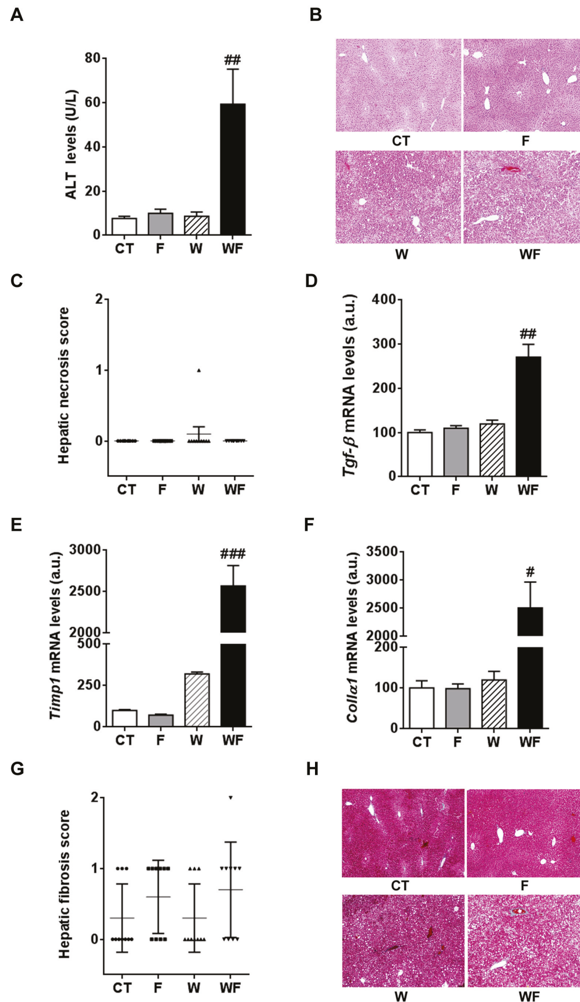

Figure 4. (**A**) Alanine aminotransferase (ALT) activity expressed as mean (U/L) ± SEM of five to six different plasma samples from Control (CT, mice fed standard solid-chow), Fructose (F, mice fed standard solid-chow plus a 15% fructose solution ad libitum), Western (W, mice fed Western solid-chow), and Western + Fructose (WF, mice fed Western solid-chow plus a 15% fructose solution ad libitum); (**B**,**C**) Histological examination of necrosis by hematoxylin and eosin staining. A representative image of liver sections from each experimental group is shown (magnification 10×). The scatter plot shows individual values of necrosis degree (0, absent; 1, <1%). The horizontal line across each group locates the mean, and bars indicate the SEM; (**D**–**F**) Bar plots showing the relative levels of specific mRNAs of fibrogenesis-related molecules, represented as mean (a.u., arbitrary units) ± SEM of five different liver samples obtained from CT, F, W and WF groups. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. W values; (**G**,**H**) Histological examination of fibrosis by Masson's trichrome acid staining. The scatter plot shows individual values of fibrosis score (0, no fibrosis; 1, portal or sinusoidal fibrosis without septa; 2, portal or sinusoidal fibrosis with rare septa). The horizontal line across each group locates the mean, and bars indicate the SEM. A representative image of liver sections from each experimental group is shown (magnification 10×).

3.4. Fructose Supplementation and a Western Diet Reduce Basal Insulin Receptor Substrate-2 (IRS-2) Expression but Do Not Impair Insulin-Stimulated Akt Phosphorylation in the Liver of LDL-R$^{-/-}$ Mice

Plasma glucose and plasma insulin levels remained essentially unaffected by the different dietary interventions (Figure 5A,B). When we calculated the insulin sensitivity index (ISI), we observed a non-significant trend to reduction in the WF group (Figure 5C). In addition, we calculated another surrogate index of insulin sensitivity, the HOMA-IR, and the results also pointed to lower insulin sensitivity, especially in the WF group compared to W, although it did not reach statistical significance (1.7 ± 0.6, 4.3 ± 3.6, 3.0 ± 1.1 and 5.9 ± 1.2, expressed as mean ± SEM, for C, F, W and WF groups). Thus, we wondered whether the establishment of an inflammatory and pro-fibrotic status in the liver could alter insulin signalling in this tissue. The basal expression of IRS-2, one of the main transducers of the insulin signal in the liver, was reduced in F and W groups vs. CT, and further diminished in WF vs. W (Figure 5D). We also determined the amount of IRS-2 phosphorylated at Ser731, as phosphorylation at this position has been related to impaired insulin signal transduction [26]. Our results showed significant increases in IRS-2 phosphorylation status in the W group compared to the CT group and in the WF group versus the W group (Figure 5E).

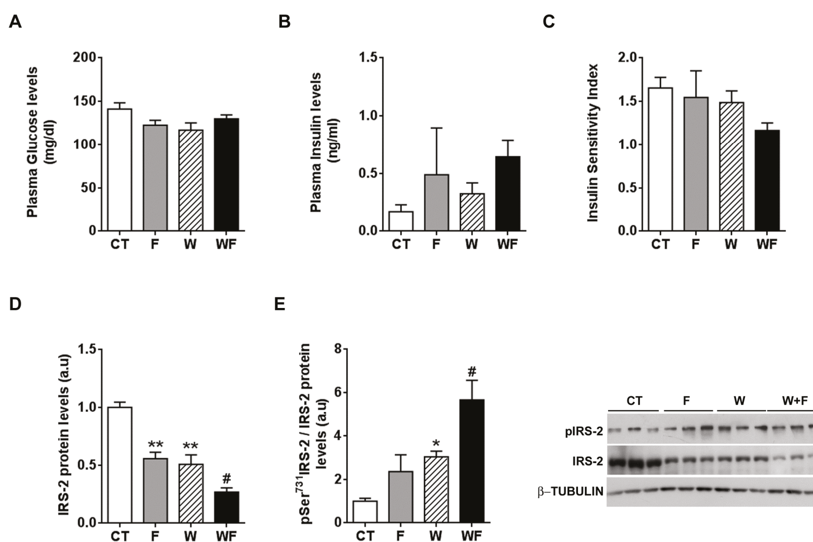

Figure 5. Insulin receptor signalling under basal conditions. Glucose (**A**) and insulin (**B**) plasma levels from Control (CT, mice fed standard solid-chow), Fructose (F, mice fed standard solid-chow plus a 15% fructose solution ad libitum), Western (W, mice fed Western solid-chow), and Western + Fructose (WF, mice fed Western solid-chow plus a 15% fructose solution ad libitum), expressed as mean ± SEM of 9–10 different samples; (**C**) Insulin Sensitivity Index (ISI), calculated as [2/(blood insulin (nM) × blood glucose (μM) + 1]; Western-blot of total and phosphorylated IRS-2 (**D**,**E**) proteins in liver samples obtained from the different experimental groups under basal conditions. Representative bands corresponding to three different mice in each group are shown; bar plots show the level of total IRS-2 expressed as the mean (a.u., arbitrary units) ± SEM of the values obtained from three to four animals, and the ratio between phosphorylated and total IRS-2. * $p < 0.05$ and ** $p < 0.01$ vs. CT values; # $p < 0.05$ vs. W values.

To further explore whether hepatic insulin sensitivity was impaired, we injected insulin (0.15 units/g body weight) in a subset of rats and 15 minutes later we assessed the phosphorylation status of several molecules related to insulin signalling: the beta subunit of the insulin receptor (IR-β),

IRS-2 and Akt. As shown in Figure 6A, insulin-induced IR-β phosphorylation tended to be lower in W and WF groups, but these differences did not reach significance. On the other hand, under acute insulin-stimulated conditions, neither the phosphorylation of IRS-2 at Ser731, nor Akt phosphorylation at both Thr308 and Ser473 positions were modified by any of the dietary interventions (Figure 6B–D).

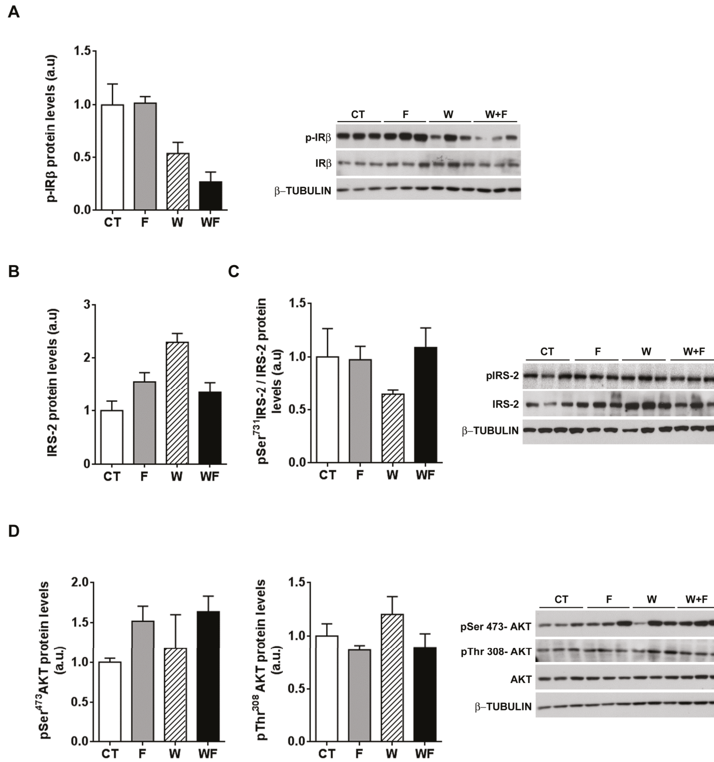

Figure 6. Insulin receptor signalling after an acute insulin challenge. Western-blot of total and phosphorylated IR-β (**A**), IRS-2 (**B**,**C**) and Akt (**D**) proteins in liver samples obtained from the different experimental groups, after exogenous insulin administration (0.15 units/g body weight). Representative bands corresponding to three different mice in each group are shown; bar plots show the level of the proteins expressed as the mean (a.u., arbitrary units) ± SEM of the values obtained from three to four animals. For IRS-2, as the corresponding total protein levels vary significantly between groups, the bar plot shows the ratio between phosphorylated and total protein levels.

4. Discussion

Here we show that consumption of a Western-type diet for 12 weeks causes an inflammatory reaction in the liver of male LDL-R$^{-/-}$ mice that is exacerbated by 15% *w*/*v* liquid fructose supplementation, which also increases fibrogenesis markers. However, vWAT is not inflamed and hepatic insulin-induced Akt phosphorylation is not hampered, despite a clear reduction in basal IRS-2 levels.

In this study we used LDL-R$^{-/-}$ mice, a model of diet-induced atherosclerosis that is also suitable for investigating the progress from hepatic steatosis to non-alcoholic steatohepatitis (NASH) [19]. We fed these mice either a standard rodent chow diet or a Western-type diet rich in cholesterol (0.21%), saturated fat (21%) and sucrose (34%) to provide a high fructose intake [18]. A high level of fructose in the diet is required to mimic all human NASH features in rodents and is typical of the diet consumed

by humans with NASH [27]. Moreover, we added liquid fructose to both diets (15% *w*/*v*), since unhealthy human diets are also characterised by a high intake of beverages sweetened with sucrose or high-fructose corn syrup (both of which contain fructose), and this has been linked to the acquisition of NAFLD and its progression to NASH and hepatic fibrosis [28,29].

Bieghs et al. [19] showed that LDL-R$^{-/-}$ mice develop sustained hepatic inflammation upon high cholesterol feeding due to the uptake of oxidized low-density lipoproteins through the CD36 receptor. Moreover, it has been shown that hepatic cholesterol accumulation is one of the main drivers of hepatic inflammation in LDL-R$^{-/-}$ mice [30]. In the present study, only mice with the highest liver cholesterol content (W and WF groups) show a clear inflammatory response in this tissue, and the response is more intense in the WF group (Supplemental Table S2 and Figure 2). This is in agreement with our previous observation of increased CD36 mRNA expression in WF-LDL-R$^{-/-}$ mice [18]. In contrast, our recent study performed in wild type C57BL/6N mice, showed no signs of hepatic inflammation despite a significant increase in hepatic cholesterol deposition in W and WF groups [17]. To compare the hepatic cholesterol deposition between wild type and knockout mice, we used data from [17,18], and expressed them as mg of cholesterol per gram protein. This is more accurate for comparison, due to the significant increase in liver weight observed in LDL-R$^{-/-}$ mice from the WF group, but not in wild-type mice. As shown in Figure 7A, the amount of cholesterol per gram of liver protein in control LDL-R$^{-/-}$ mice is already as high as the amount observed in wild type mice fed with the Western-type diet, and W and WF LDL-R$^{-/-}$ mice more than doubled the hepatic cholesterol compared to the same dietary groups in wild type mice. The presence of hepatic inflammation in LDL-R$^{-/-}$ but not in wild type mice may thus be related to the larger cholesterol accumulation in the liver of knockout mice even when fed a healthy control diet. In support of this hypothesis, we found significant correlations between total cholesterol content in the livers of LDL-R$^{-/-}$ mice and the hepatic expression of the inflammatory markers *tlr4*, *tnfα*, *mcp-1*, *f4/80*, as well as the inflammasome components *nlrp3*, *caspase-1* and *asc* (Figure 7B–H).

However, other pathways besides cholesterol accumulation may be involved in the onset of hepatic inflammation and its exacerbation by fructose addition. It has been reported that liquid fructose supplementation (30% *w*/*v*) in mice increases intestinal permeability and thus allows bacterial endotoxins to enter portal blood, which then leads to the development of liver TLR4-mediated inflammation [31,32]. Our results show that liquid fructose supplementation in the LDL-R$^{-/-}$ model does not seem to increase blood endotoxins, as revealed by unchanged plasma levels of LBP, a biomarker of circulating lipopolysaccharide (Figure 3B). This lack of effect can be attributed to the use of a different strain or the lower concentration of fructose solution used in our study (15 vs. 30% *w*/*v*). However, we show here that plasma LBP increases significantly in mice fed a Western-type diet compared to the chow-fed mice (Figure 3B). This suggests that the increase in intestinal permeability to bacterial toxins in our model may be caused by some component of the Western diet. Thus in WF mice, which ingest a smaller amount of solid food than the W group [18], the amount of plasma LBP is also somewhat reduced, albeit not significantly (Figure 3B). Gut-derived bacterial components act as a first signal to prime the inflammasome, through binding to TLRs and subsequent NFκB activation leading to the upregulation of NLRP3 transcription [25]. In accordance with the higher LBP levels, our results show that the Western-type diet increases *tlr4* mRNA expression and that fructose supplementation enhances this effect. However, significant NFκB activation (Figure 3C) and *nlrp3* induction (Figure 3A) are only observed in the WF group, suggesting that fructose or the Western-type diet alone do not suffice to fully prime the inflammasome. Activation of the TLR4-NFκB pathway enables a second, non-microbial-derived signal to activate the NLRP3 inflammasome [25]. It has been shown that cholesterol crystals activate the NLRP3 inflammasome [33], and as we have mentioned before the highest hepatic cholesterol levels are only achieved when fructose is added to a Western-type diet. Thus, it is plausible to suggest that fructose added to a healthy dietary substrate is not potent enough to activate the inflammasome, but it may act as a second step contributing to full inflammasome activation in the WF group.

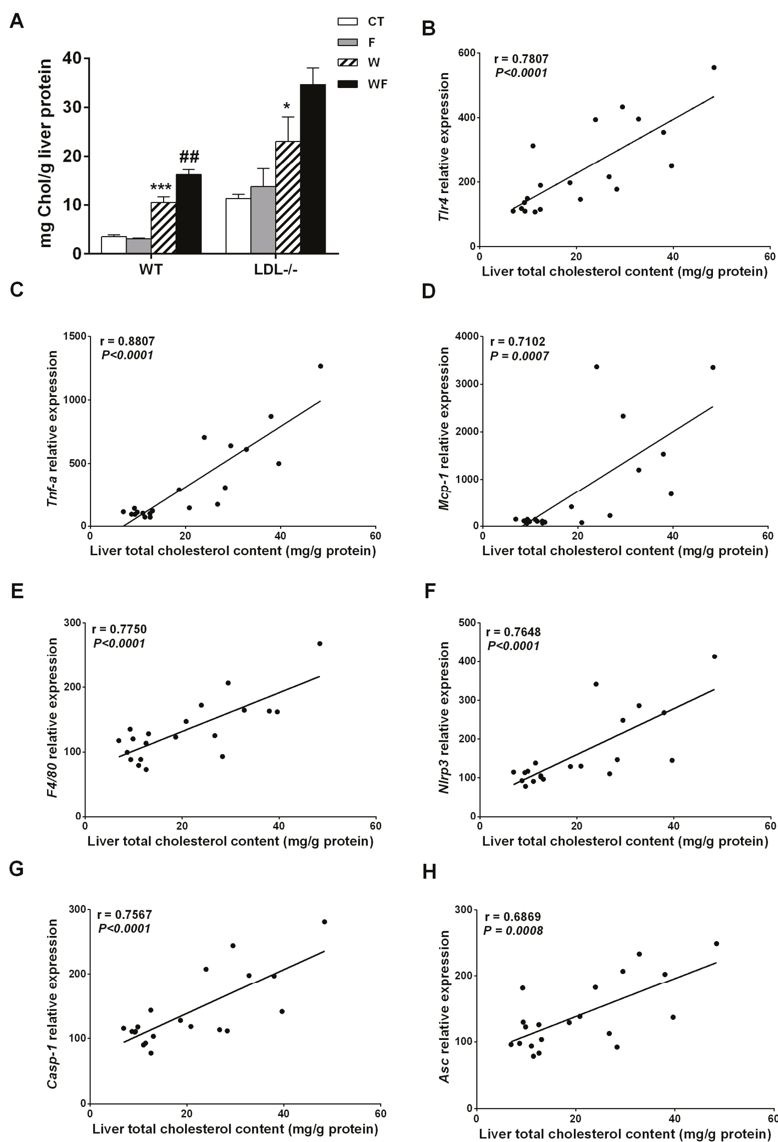

Figure 7. (**A**) Liver cholesterol levels from wild-type (WT) and LDL-R$^{-/-}$ mice fed standard solid-chow (CT), standard solid-chow plus a 15% fructose solution ad libitum (F), Western solid-chow (W), and Western solid-chow plus a 15% fructose solution ad libitum (WF), expressed as mean ± SEM of 9–10 animals; Data were obtained from [17,18]. Correlation between liver cholesterol content and mRNA expression of pro-inflammatory molecules including: (**B**) toll-like receptor 4 (*tlr4*); (**C**) tumor necrosis factor (*TNF-α*); (**D**) monocyte chemoattractant protein-1 (*mcp-1*); (**E**) f4/80; (**F**) NLR family pyrin domain containing 3 (*nlrp3*); (**G**) caspase-1 (*casp1*) and (**H**) apoptosis-associated speck-like protein containing a caspase-recruitment domain (*asc*). Statistical analysis was performed using two tailed Pearson or Spearman correlation. R indicates correlation coefficient.

Inflammasome activation leads to the induction of *mcp-1* and thus to the recruitment of inflammatory cells into the damaged tissue [25]. Accordingly, although *mcp-1* and *f4/80* mRNA levels are gradually increased by the dietary interventions, and the increase is maximal in the group in which inflammasome is fully activated, i.e., the WF group (Figure 2). Inflammasome activation also stimulates stellate hepatic cells through increased IL-1 secretion and leads to fibrosis [25]. This is also consistent with our results, since the expression of the critical inducers of fibrogenesis (*tgfβ* and *timp-1*), as well as the expression of one of the predominant collagens in the fibrotic liver (*collα1*), are only significantly increased in livers from the WF group (Figure 4D–F). Liver fibrosis is a dynamic process, where progression to advanced fibrotic stages is characterized by increased synthesis and decreased degradation of extracellular matrix (ECM) proteins, whereas the opposite may lead to regression or reversal [34,35]. Several studies in humans and animal models suggest a prominent role for TIMP-1 in this process, as TIMP-1 up-regulation by inflammatory cytokines, especially TGFβ, shifts the balance toward ECM synthesis and fibrogenesis [35,36]. TIMP-1 not only prevents the degradation of the accumulated ECM by blocking the function of matrix metalloproteases, thus opposing to fibrosis reversal, but also inhibits the apoptosis of activated hepatic stellate cells, the main cellular source of type I collagen [37]. Thus, although histological signs of overt hepatic fibrosis are still absent in our model (Figure 4G,H), the marked induction of TIMP-1 in the WF group suggest that with longer exposure to this WF diet fibrosis would continue unopposed, leading to progression to advanced fibrotic stages.

The presence of hepatic steatosis [18] and the inflammatory and pro-fibrogenic status in the liver of LDL-R$^{-/-}$ mice caused by the combined effects of fructose and the Western diet prompted us to examine the effects of the dietary interventions on the hepatic insulin signalling cascade. Although plasma glucose and insulin levels do not vary significantly between groups, whole-body insulin sensitivity (measured by the ISI) tends to decrease in the WF group. Moreover, under basal conditions, the amount of total IRS-2 is reduced and phosphorylation of IRS-2 at Ser731 is increased across the different dietary groups, which suggest an impairment of hepatic insulin receptor signalling cascade (Figure 5). However, after an insulin challenge total IRS-2 expression is not reduced and its phosphorylation status does not vary significantly. In addition, the phosphorylation of Akt at Thr308 and Ser473, which lead to full Akt activation after insulin stimulation [38–40], is not impaired by any of the dietary regimes (Figure 6). These results suggest that despite there is some degree of impairment in insulin receptor signalling under basal conditions, when insulin levels are high this deficit is overcome and signal transduction proceeds correctly.

Our results showing inflammation and signs of progression to fibrosis contrasts with the prevalent theory that links these phenomena with IR [2–5]. However, it has been suggested that insulin sensitivity is not reduced when the inflammation is restricted to the liver compartment and does not affect adipose tissue [1,11,41]. Our results are in accordance with this hypothesis, as LDL-R$^{-/-}$ mice show no signs of inflammation in vWAT (Figure 1), whereas in our previous study in wild type mice we observed inflammation in vWAT but not in the liver, and whole-body insulin sensitivity was significantly reduced [17].

In contrast to our results, Subramanian et al. [30] found a marked inflammatory reaction in epididymal adipose tissue from LDL-R$^{-/-}$ mice that were fed a diabetogenic diet with 0.15% cholesterol, which was linked to whole-body IR. Although the amount of cholesterol in the Western-type diet was even greater in our study (0.21%), we used a shorter feeding period (12 weeks instead of 24 weeks). Long-term studies (5.5 months) also show that a Western-type diet similar to the one used in the present study causes IR with hyperglycaemia and hyperinsulinemia [42]. Therefore, we cannot rule out the possibility that longer exposure to the Western-type diet, with or without liquid fructose supplementation, could also induce adipose tissue inflammation and IR. However, in a recent study with a relatively short duration (16–20 weeks), LDL-R$^{-/-}$ mice fed diets that provided more calories from fat (58%–60%) and fewer calories from carbohydrate (20%–28%) than our Western-type diet (40 kcal% fat and 43 kcal% carbohydrate) showed inflammation in both liver and adipose tissue,

accompanied by IR and impaired glucose tolerance [43]. Therefore, both the duration of the study and the type of nutrients provided by the diet determine the metabolic response.

Taken together, our results suggest that liquid fructose supplementation in a Western-type diet in LDL-R$^{-/-}$ mice aggravates the inflammatory response in the liver and initiates fibrogenesis, but the lack of inflammation in vWAT probably delays the manifestation of glucose intolerance and impaired hepatic insulin signalling.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/3/278/s1, Table S1: Primers used for RT-PCR., Table S2: Zoometric parameters of LDLR$^{-/-}$ mice exposed to four different dietary regimes.

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Author Contributions: Gemma Sangüesa and Miguel Baena performed the experiments and analysed the data. Natalia Hutter and José Carlos Montañés ran the animal study and contributed to western blot and PCR experiments. Rosa María Sánchez helped in data interpretation and reviewed the manuscript. Núria Roglans contributed to western blot experiments and prepared the figures. Juan Carlos Laguna and Marta Alegret designed the experiments, supervised data analysis and wrote the manuscript.

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An In Vivo Magnetic Resonance Spectroscopy Study of the Effects of Caloric and Non-Caloric Sweeteners on Liver Lipid Metabolism in Rats

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† In memory of Professor Klaas Nicolay, who deceased before publication of this work. His expertise was crucial for the design and conduct of this study.

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Abstract: We aimed to elucidate the effects of caloric and non-caloric sweeteners on liver lipid metabolism in rats using in vivo magnetic resonance spectroscopy (MRS) and to determine their roles in the development of liver steatosis. Wistar rats received normal chow and either normal drinking water, or solutions containing 13% (w/v) glucose, 13% fructose, or 0.4% aspartame. After 7 weeks, in vivo hepatic dietary lipid uptake and de novo lipogenesis were assessed with proton-observed, carbon-13-edited MRS combined with ^{13}C-labeled lipids and ^{13}C-labeled glucose, respectively. The molecular basis of alterations in hepatic liver metabolism was analyzed in detail ex vivo using immunoblotting and targeted quantitative proteomics. Both glucose and fructose feeding increased adiposity, but only fructose induced hepatic lipid accumulation. In vivo MRS showed that this was not caused by increased hepatic uptake of dietary lipids, but could be attributed to an increase in de novo lipogenesis. Stimulation of lipogenesis by fructose was confirmed by a strong upregulation of lipogenic enzymes, which was more potent than with glucose. The non-caloric sweetener aspartame did not significantly affect liver lipid content or metabolism. In conclusion, liquid fructose more severely affected liver lipid metabolism in rats than glucose, while aspartame had no effect.

Keywords: obesity; fatty liver disease; hepatic steatosis; carbohydrate; glucose; fructose; aspartame

1. Introduction

In 2013, an estimated 2.1 billion people were overweight or obese compared with 857 million people in 1980 [1]. The rise in obesity is associated with an increased prevalence of non-alcoholic fatty liver disease (NAFLD), which can progress into non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma [2–4]. The increased consumption of simple carbohydrates has been identified as one of the contributing factors to the obesity epidemic [5]. Especially the chronic

consumption of fructose, as opposed to glucose, has been linked to the development of obesity, insulin resistance, dyslipidemia, and hepatic steatosis [6–10].

Unlike glucose, fructose is mainly metabolized by the liver. The metabolism of fructose to triose phosphate bypasses phosphofructokinase, a key regulatory step of glycolysis, allowing unregulated entry of fructose into glycolysis independent of hepatic energy status. This leads to an excess production of triose phosphates, which serve as precursors for de novo lipogenesis [9,11,12]. Additionally, fructose consumption causes activation of transcription factors regulating de novo lipogenesis, i.e., sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP), resulting in increased expression of lipogenic enzymes such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) [9–11,13,14]. ACC catalyzes the conversion of acetyl-CoA into malonyl-CoA, providing the building blocks for fatty acid synthesis. At the same time, increased levels of malonyl-CoA as a result of fructose-induced overexpression of ACC suppresses fatty acid β-oxidation through inhibition of carnitine palmitoyl transferase 1 (CPT1) [15]. In addition, fructose suppresses the oxidation of fatty acids by decreasing the activity of peroxisome proliferator-activated receptor α (PPARα) [14,16–18]. Thus, the stimulation of de novo lipogenesis and the inhibition of fatty acid β-oxidation likely contribute to fructose-induced hepatic lipid accumulation. Moreover, fructose feeding has been shown to increase the intestinal production of apolipoprotein B48 (apoB48) [19–21], which may promote the absorption of dietary lipids from the intestine, resulting in an increased influx of diet-derived lipids into the liver. However, the relative importance of the different pathways leading to fructose-induced hepatic lipid accumulation remains unknown.

Non-caloric sweeteners simulate the sweet taste of sugars without the calories and are of great value in the fight against obesity [22]. Some studies showed beneficial effects of the consumption of non-caloric sweeteners by inducing weight loss and reducing the risk factors for metabolic syndrome [23–25]. In contrast, others demonstrated a correlation between the use of non-caloric sweeteners and weight gain and increased risk of type 2 diabetes and insulin resistance [26–29]. The most controversial and most used non-caloric sweetener is aspartame (methyl L-α-aspartyl-L-phenylalaninate). Aspartame consumption has been shown to increase fasting blood glucose levels and induce glucose intolerance in rats and mice, and these effects have been attributed to compositional and functional changes in gut microbiota [26,30,31]. However, data on the effects of aspartame on hepatic lipid metabolism are greatly lacking.

The aim of this study was to investigate the effects of caloric (glucose and fructose) and non-caloric (aspartame) sweeteners on liver lipid content and metabolism in vivo in rats. We recently developed a method that combines localized proton-observed, carbon-13-edited magnetic resonance spectroscopy (^{1}H-[^{13}C] MRS) with the oral administration of ^{13}C-labeled lipids to determine dietary lipid uptake in vivo [32,33]. Here we introduce a new variant of this approach, in which we administer ^{13}C-labeled glucose instead of ^{13}C-labeled lipids. In this case, the ^{13}C-labeled liver lipids detected by ^{1}H-[^{13}C] MRS originate from the conversion of ^{13}C-labeled glucose to ^{13}C-labeled lipids through de novo synthesis. This novel application of ^{1}H-[^{13}C] MRS with the oral administration of ^{13}C-labeled glucose thus allows in vivo assessment of the contribution of de novo lipogenesis to hepatic lipid accumulation. In the present study, we applied both methods, i.e., ^{1}H-[^{13}C] MRS with the administration of ^{13}C-labeled lipids and ^{1}H-[^{13}C] MRS with the administration of ^{13}C-labeled glucose, to determine in vivo dietary lipid uptake and de novo lipogenesis, respectively, in the livers of rats receiving either normal drinking water, or 13% (*w*/*v*) glucose, 13% (*w*/*v*) fructose, or 0.4% (*w*/*v*) aspartame in their drinking water, for a period of 7 weeks. The molecular basis of alterations in hepatic liver metabolism was analyzed in detail ex vivo using immunoblotting and targeted quantitative proteomics.

2. Materials and Methods

2.1. Animals and Diets

Adult male Wistar rats (350 ± 2 g, 10–11 weeks of age, n = 60; Charles River Laboratories, The Netherlands) were housed in pairs in individually ventilated cages with corn cob bedding and standard cage enrichment at 20 °C and 50% humidity on a 12 h light-dark cycle. All animals received normal chow (9 energy percent (En%) from fat, 67 En% from carbohydrates, 24 En% from protein; R/M-H diet, Ssniff Spezialdiäten GmbH, Soest, Germany) for the duration of the study. After one week of acclimatization, the rats were divided into four groups receiving either normal drinking water (CON), or 13% (w/v) glucose (GLU), 13% (w/v) fructose (FRUC), or 0.4% (w/v) aspartame (ASP) in their drinking water. The animals had ad libitum access to food and liquids. Body weight and food and drink intake were determined weekly. The rats received the diets for a period of 7 weeks, after which each dietary group was divided into two subgroups: experimental group 1 (n = 9 per diet group) for MRS measurements to determine dietary lipid uptake and for an oral glucose tolerance test (OGTT), and experimental group 2 (n = 6 per diet group) for MRS to determine de novo lipogenesis and for hepatic biochemical analyses. Blood samples were taken from the vena saphena before each MRS experiment and were collected in paraoxon-coated capillaries to prevent lipolysis. The samples were centrifuged at 1000× g for 10 min and plasma was frozen in liquid nitrogen and stored at −80 °C for later analysis. All animal experiments were reviewed and approved by the Animal Ethics Committee of Maastricht University (DEC-UM, Maastricht, The Netherlands; project number: 2013-011; date of approval: 20 March 2013).

2.2. MRS Experiments

To determine total (^{12}C + ^{13}C) intrahepatocellular lipid (IHCL) content and natural abundance ^{13}C enrichment of IHCL in liver after 7 weeks of diet, all animals (n = 15 per diet group) were subjected to baseline ^{1}H-[^{13}C] MRS measurements in a fed condition.

Two days later, rats from experimental group 1 received 1.5 g [U-^{13}C] labeled algal lipid mixture (^{13}C enrichment > 98%; fatty acid composition: 53% palmitic acid, 9% palmitoleic acid, 28% oleic acid, and 6% linoleic acid; Buchem B.V., Apeldoorn, The Netherlands) per kg body weight by oral gavage. The following 4 h the rats remained fasted, after which ^{1}H-[^{13}C] MRS experiments were performed to determine ^{13}C-enriched IHCL concentrations.

Starting on the day after baseline MRS measurements, rats from experimental group 2 were administered 3.33 g [U-$^{13}C_6$]glucose (^{13}C enrichment >98%; Buchem B.V., Apeldoorn, The Netherlands) per kg body weight by oral gavage, two times a day for a period of 5 days. The following day, ^{1}H-[^{13}C] MRS experiments were performed in a fed condition to determine ^{13}C-enriched IHCL concentrations. After these MRS measurements, animals from experimental group 2 were euthanized by incising the vena cava inferior, and the median lobe of the liver was excised and stored at −80 °C.

^{1}H-[^{13}C] MRS experiments were performed on a 7 T horizontal Bruker MR system (Bruker, Ettlingen, Germany), as described previously [32]. During the MRS experiments, animals were anaesthetized using 1.5–2.5% isoflurane (IsoFlo®; Abbott Laboratories Ltd., Maidenhead, Berkshire, UK). Total (^{12}C + ^{13}C) and ^{13}C-labeled IHCL levels are presented as a percentage of the unsuppressed water signal measured in the same voxel. The relative ^{13}C enrichment determined at baseline was used to correct the ^{13}C-enriched IHCL levels after administration of ^{13}C-labeled lipids or glucose for natural abundance of ^{13}C.

2.3. Oral Glucose Tolerance Test

In experimental group 1, two days after the last MRS measurements, an OGTT was performed (n = 9 per diet group). After an overnight fast, rats received 1 mg/g body weight glucose orally. Blood samples were taken from the vena saphena just before and at 15, 30, 45, 60, 90, and 120 min after glucose administration. Plasma glucose concentration was determined using a HemoCue Glucose 201 RT Analyzer

(HemoCue AB, Ängelholm, Sweden), while plasma insulin concentration was analyzed using the Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden). Areas under the glucose (AUC_g) and insulin (AUC_i) curves were calculated. Directly after the OGTT, animals from experimental group 1 were euthanized by incising the vena cava inferior, and the median lobe of the liver was excised and stored at −80 °C.

2.4. Plasma and Tissue Analyses

Plasma triglyceride (TG) and alanine aminotransferase (ALT) were determined using serum TG determination kit (Sigma-Aldrich, Zwijndrecht, The Netherlands) and EnzyChrom™ ALT assay kit (Bio-Connect Diagnostics, Huissen, The Netherlands), respectively, following the manufacturer's instructions. Liver malonyl-CoA content was determined as described in [34].

2.5. Determination of Glycogen Content in Liver

A 200-mg snap-frozen liver sample was homogenized in 10 mL of 25 mM citrate solution (pH 4.2) containing 2.5 g/L NaF and centrifuged at 10,000× *g* for 8 min to remove debris. Glycogen content in the supernatant was determined using EnzyChrom™ glycogen assay kit (Bio-Connect Diagnostics, Huissen, The Netherlands) according to the manufacturer's instructions.

2.6. Glycolytic Enzyme Activities

Pieces of snap-frozen liver were powdered in liquid nitrogen and 10% (*w*/*v*) homogenates were prepared in ice cold phosphate-buffered saline (PBS), pH 7.4. Homogenates were sonicated for 30 s in the pulse mode (pulse duration 1 s, interval between the pulses 1 s, amplitude 20%) on ice, followed by 10 min centrifugation at 1000× *g*, 4 °C. The supernatant was used for spectrophotometric determination of enzyme activities of phosphoglucose-isomerase [35], phosphoglycerate kinase [36], and pyruvate kinase [37]. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA) and enzyme activities were expressed per mg liver protein.

2.7. Western Blot Analysis

Protein expression levels of ACC, FAS, SREBP-1c, ChREBP, and PPARα were determined by immunoblotting as described in the Supporting Information.

2.8. Targeted Quantitative Mitochondrial Proteomics

Selected 47 mitochondrial proteins involved in substrate transport, oxidative phosphorylation, fatty acid β-oxidation, and tricarboxylic acid (TCA) cycle were quantified in liver samples using targeted quantitative proteomics as described in [38].

2.9. Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). The statistical significance of differences among the diet groups was assessed using one-way analysis of variance (ANOVA) with Tukey honest significant difference (HSD) post-hoc analyses. Differences between the two experimental groups were determined by univariate ANOVA with Tukey HSD post-hoc analyses. Statistical analyses were performed using IBM SPSS statistics 22 software package (SPSS, Inc.; Chicago, IL, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. Caloric Sweeteners Increase Adiposity without an Effect on Body Weight

Before the start of the diets, body weights were not significantly different between groups (Table 1). During the diet period, body weights increased similarly among the different groups (Figure 1A) and

after 7 weeks of diet body weights were not significantly different (Table 1). However, both epididymal and perirenal fat pads of rats in the caloric sweetener groups GLU and FRUC were increased compared with rats in the CON and ASP groups (Table 1). Food intake was lower in the GLU and FRUC groups compared with CON and ASP, but their drink intake was higher, resulting in a higher total energy intake in GLU and FRUC compared with CON and ASP groups (Figure 1B, Table 1). Furthermore, animals in the FRUC group consumed more food, but had a lower drink intake compared with GLU animals, resulting in a slightly lower total energy intake in FRUC compared with GLU.

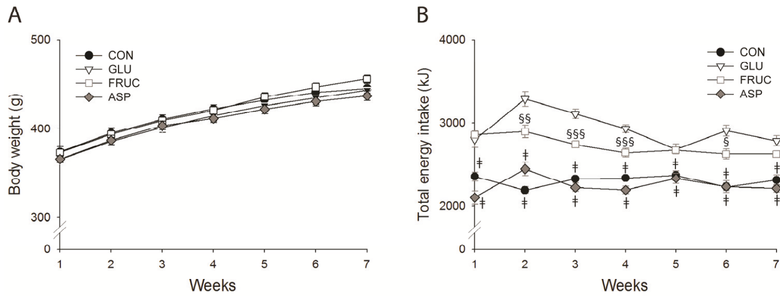

Figure 1. Body weight (**A**) and total energy intake (**B**) of rats receiving normal water (CON), a 13% (*w*/*v*) glucose solution (GLU), a 13% (*w*/*v*) fructose solution (FRUC), or a 0.4% (*w*/*v*) aspartame solution (ASP), determined weekly (n = 15 per diet group). Data are expressed as means ± standard error of the mean (SEM). ‡ $p < 0.001$ vs. GLU and FRUC; § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ vs. GLU.

In the FRUC group, plasma TG concentrations were higher compared with the ASP group (Table 1). Moreover, plasma ALT levels were higher in FRUC animals compared with CON and GLU animals (Table 1).

Liver weight and liver glycogen concentrations were analyzed separately per experimental group (Table 1), because liver tissue was harvested under different conditions, i.e., fasted versus fed. In both experimental groups, FRUC animals had higher liver weights compared with GLU and ASP, while there were no significant differences in liver glycogen content between the diet groups. When comparing between the two experimental groups, liver weight and liver glycogen content were higher in experimental group 2 compared with experimental group 1, which is likely explained by the fasted state of animals in experimental group 1.

3.2. Both Caloric and Non-Caloric Sweeteners Affect Whole-Body Glucose Homeostasis

Whole-body glucose tolerance was assessed with an OGTT (Table 2). There were no significant differences in fasting plasma glucose or AUC_g among the different diet groups. Fasting plasma insulin, on the other hand, was higher in ASP and tended to be higher in GLU (p = 0.051) and FRUC (p = 0.127) compared with CON. AUC_i and the product of AUC_g and AUC_i also tended to be increased in GLU, FRUC, and ASP compared with CON, although this only reached statistical significance in the case of FRUC.

Table 1. Animal characteristics.

	CON	GLU	FRUC	ASP
Start body weight (g)	354 ± 6	347 ± 3	351 ± 3	348 ± 3
End body weight (g)	445 ± 5	444 ± 8	456 ± 5	437 ± 6
Body weight gain (g)	92 ± 4	97 ± 6	105 ± 4	89 ± 4
Food intake (kJ/week)	2300 ± 25	1170 ± 24 ***,†††	1447 ± 36 ***,†††,§§§	2232 ± 35
Food intake (g/week)	180 ± 2	91 ± 2 ***,†††	113 ± 3 ***,†††,§§§	174 ± 3
Drink intake (kJ/week)	NA	1757.9 ± 48.7 †††	1274.4 ± 44.9 †††,§§§	13.4 ± 0.5
Drink intake (mL/week)	177 ± 4	808 ± 22 ***,†††	586 ± 21 ***,†††,§§§	201 ± 7
Total energy intake (kJ/week)	2300 ± 25	2927 ± 35 ***,†††	2721 ± 20 ***,†††,§§§	2246 ± 36
Amount sweetener (g/kg BW/day)	NA	36.87 ± 1.25 †††	26.16 ± 1.00 †††,§§§	0.28 ± 0.01
Epididymal fat (g) ($n = 36$)	5.8 ± 0.2	7.5 ± 0.4 **,††	7.1 ± 0.3 *,†	5.6 ± 0.3
Perirenal fat (g) ($n = 36$)	5.8 ± 0.4	9.5 ± 0.6 ***,†††	9.0 ± 0.5 ***,†††	5.8 ± 0.4
Plasma TG (mM)	1.05 ± 0.10	1.06 ± 0.10	1.23 ± 0.04 †	0.87 ± 0.04
Plasma ALT (U/L)	24.94 ± 2.35	21.71 ± 3.11	51.21 ± 8.00 **,§§	35.44 ± 4.32
Experimental group 1 ($n = 9$ per diet group)				
Liver weight (g)	10.63 ± 0.14	9.97 ± 0.16	11.39 ± 0.22 †,§§	10.17 ± 0.25
Liver glycogen (mg/g ww)	87 ± 10	78 ± 7	75 ± 4	67 ± 6
Experimental group 2 ($n = 6$ per diet group)				
Liver weight (g)	12.92 ± 0.16 $^{\#\#\#}$	13.08 ± 0.64 $^{\#\#\#}$	16.25 ± 0.59 $^{***,\dagger\dagger\dagger,\S\S\S,\#\#\#}$	13.23 ± 0.21 $^{\#\#\#}$
Liver glycogen (mg/g ww)	96 ± 3	118 ± 8 $^{\#\#\#}$	117 ± 8 $^{\#\#\#}$	115 ± 8 $^{\#\#\#}$
Liver malonyl-CoA (nmol/g ww)	58.37 ± 0.97	62.44 ± 2.70	68.05 ± 2.32 *,††	56.31 ± 1.82

Data are expressed as means ± standard error of the mean (SEM) of 15 animals per diet group (unless stated otherwise). Experimental group 1 was sacrificed after an overnight fast, while experimental group 2 was sacrificed in a fed condition. CON, normal water control; GLU, 13% (*w*/*v*) glucose; FRUC, 13% (*w*/*v*) fructose; ASP, 0.4% (*w*/*v*) aspartame; NA, not applicable; BW, body weight; TG, triglycerides; ALT, alanine aminotransferase; ww, wet weight. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CON; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. ASP; §§ $p < 0.01$, §§§ $p < 0.001$ vs. GLU; $^{\#\#\#}$ $p < 0.001$ vs. experimental group 1.

Table 2. Plasma glucose and insulin concentrations during oral glucose tolerance test (OGTT).

	CON			GLU			FRUC			ASP		
Fasting glucose (mM)	4.87	±	0.21	4.44	±	0.27	4.35	±	0.10	5.22	±	0.40
AUC_g (mM·h)	13.67	±	0.39	15.07	±	0.55	14.89	±	0.43	13.87	±	0.46
Fasting insulin (pM)	267	±	64	605	±	97	500	±	81	573	±	61 *
AUC_i (pM·h)	454	±	64	576	±	75	614	±	54	562	±	52
$AUG_g \cdot AUC_i$ (mM·h·pM·h)	5462	±	672	8640	±	1151	9274	±	1031 *	7824	±	770

Data are expressed as means ± SEM of nine animals per diet group. CON, normal water control; GLU, 13% (*w*/*v*) glucose; FRUC, 13% (*w*/*v*) fructose; ASP, 0.4% (*w*/*v*) aspartame; AUC_g, area under the glucose curve; AUC_i, area under the insulin curve. * $p < 0.05$ vs. CON.

3.3. Fructose Stimulates Hepatic De Novo Lipogenesis

Next, we assessed the effects of caloric and non-caloric sweeteners on hepatic lipid metabolism in vivo using ^{1}H-[^{13}C] MRS (Figure 2). Figure 3 shows total IHCL content and ^{13}C-enriched IHCL content after the administration of [U-^{13}C] algal lipid mixture or [U-$^{13}C_6$]glucose. After 7 weeks of diet, IHCL levels were significantly higher in FRUC animals compared with CON and ASP (Figure 3A). Four hours after the administration of [U-^{13}C] algal lipid mixture, levels of ^{13}C-enriched IHCL were not significantly different among the groups, showing that dietary lipid uptake in the liver was not significantly affected by glucose, fructose, or aspartame consumption (Figure 3B). After 5 days of [U-$^{13}C_6$]glucose administration, on the other hand, ^{13}C-enriched IHCL was significantly higher in FRUC compared with all other groups, demonstrating increased de novo lipogenesis in the liver upon fructose feeding (Figure 3C).

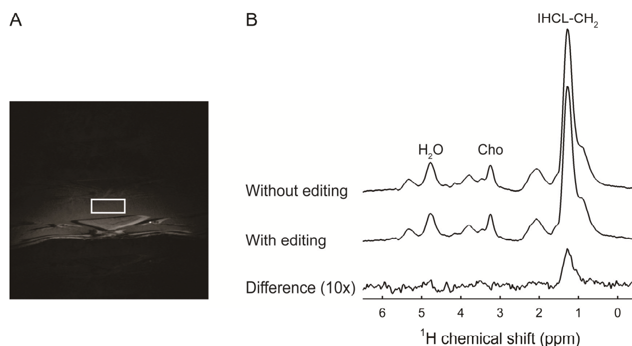

Figure 2. (**A**) T_1-weighted transversal image of the abdomen of a rat receiving a 13% (*w*/*v*) fructose solution. The positioning of a 5 × 2 × 4 mm^3 voxel in the median lobe of the liver for ^{1}H-[^{13}C] magnetic resonance spectroscopy (MRS) is indicated by the white square; (**B**) ^{1}H-[^{13}C] MRS spectra from the voxel in panel A. Spectra were acquired after 5 days of [U-$^{13}C_6$]glucose administration. Spectra without ^{13}C editing, with ^{13}C editing, and the calculated difference spectrum containing only ^{13}C-coupled ^{1}H resonances (10× magnification) are shown. Peak annotations: Cho, choline; IHCL, intrahepatocellular lipids. Total IHCL content was quantified from the spectrum without ^{13}C editing and ^{13}C-enriched IHCL content was determined from the difference spectrum.

Protein expression levels of key lipogenic enzymes ACC and FAS were not affected in the ASP group, but were increased in livers from the GLU and FRUC groups compared with the CON group, and the effect was stronger in FRUC compared with GLU (Figure 4A,B). Furthermore, fructose but not glucose feeding significantly increased the hepatic concentration of the ACC product malonyl-CoA compared to the CON group (Table 1). Total hepatic protein levels of SREBP-1c were only mildly affected in livers of GLU and FRUC animals, as indicated by a slight upregulation of SREBP-1c

precursor compared to the CON group without an effect on cleaved SREBP-1c (Figure 4E,F). Aspartame feeding resulted in lower protein levels of cleaved SREBP-1c compared to all other experimental groups. Total protein levels of ChREBP were increased in response to glucose and fructose but not aspartame feeding compared to the CON group (Figure 4D). The strongest induction was observed in the GLU group. Upregulation of ChREBP protein levels was accompanied by increased activities of glycolytic enzymes phosphoglucose-isomerase (Figure 5A) and, even more notably, pyruvate kinase (Figure 5C) in livers from GLU and FRUC animals, with the strongest effects observed in the FRUC group. The enzyme activity of phosphoglycerate kinase (Figure 5B) tended to be higher in FRUC compared with CON (p = 0.056). The activities of glycolytic enzymes were not significantly affected by aspartame feeding. The protein levels of PPARα were slightly decreased in GLU but not FRUC and ASP groups compared with CON (Figure 4C).

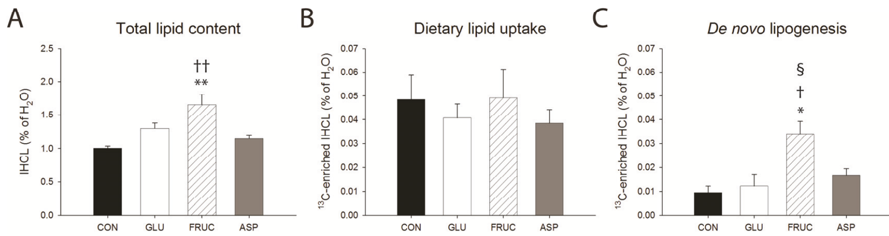

Figure 3. (**A**) Total lipid content at baseline (n = 15 per diet group); (**B**) ^{13}C-enriched IHCL 4 h after the oral administration of [U-^{13}C] algal lipid mixture (dietary lipid uptake; n = 9 per diet group); and (**C**) ^{13}C-enriched IHCL after 5 days of oral administration of [U-$^{13}C_6$]glucose (de novo lipogenesis; n = 6 per diet group), in rats receiving normal water (CON), a 13% (w/v) glucose solution (GLU), a 13% (w/v) fructose solution (FRUC), or a 0.4% (w/v) aspartame solution. Data are expressed as means ± SEM. * $p < 0.01$, ** $p < 0.001$ vs. CON; † $p < 0.05$, †† $p < 0.01$ vs. ASP; § $p < 0.05$ vs. GLU.

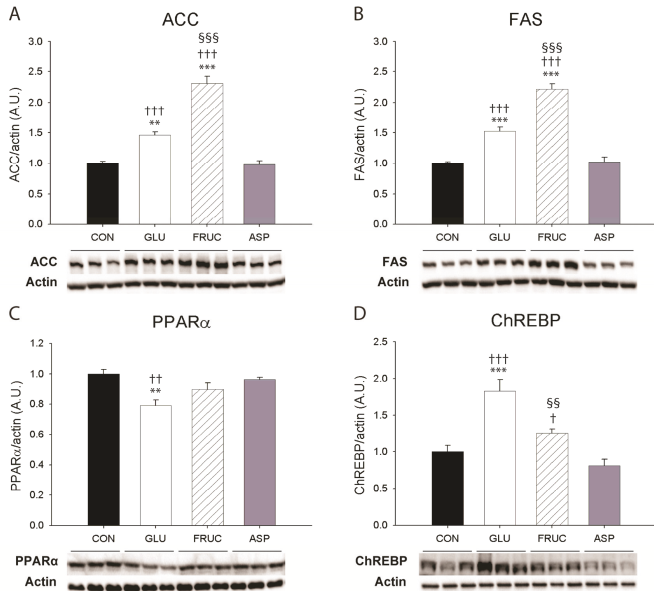

Figure 4. *Cont.*

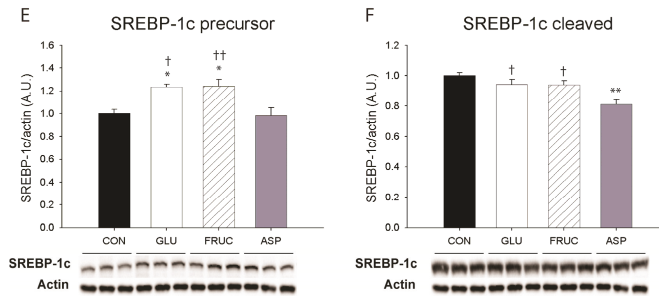

Figure 4. Protein expression levels of (**A**) acetyl-CoA carboxylase (ACC); (**B**) fatty acid synthase (FAS); (**C**) peroxisome proliferator-activated receptor α (PPARα); (**D**) carbohydrate-responsive element-binding protein (ChREBP); (**E**) 128 kDa precursor sterol regulatory element-binding protein-1c (SREBP-1c); and (**F**) 65 kDa cleaved SREBP-1c, in livers of rats receiving normal water (CON), a 13% (*w*/*v*) glucose solution (GLU), a 13% (*w*/*v*) fructose solution (FRUC), or a 0.4% (*w*/*v*) aspartame solution (n = 6 per diet group). All data were normalized to β-actin expression levels and are expressed relative to the controls (CON). Data are expressed as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CON; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. ASP; §§ $p < 0.01$, §§§ $p < 0.001$ vs. GLU.

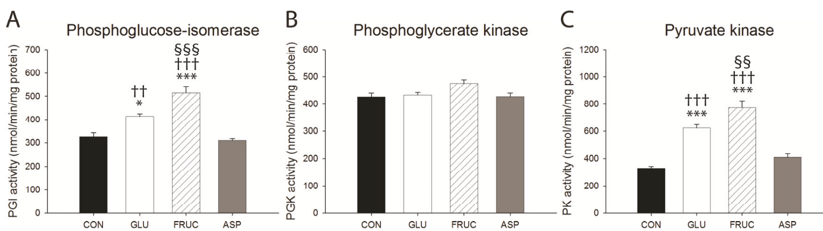

Figure 5. Glycolytic enzyme activities in liver. Activity of (**A**) phosphoglucose-isomerase (PGI); (**B**) phosphoglycerate kinase (PGK) and (**C**) pyruvate kinase (PK), in livers of rats receiving normal water (CON), a 13% (*w*/*v*) glucose solution (GLU), a 13% (*w*/*v*) fructose solution (FRUC), or a 0.4% (*w*/*v*) aspartame solution (n = 6 per diet group). Data are expressed as means ± SEM. * $p < 0.05$, *** $p < 0.001$ vs. CON; †† $p < 0.01$, ††† $p < 0.001$ vs. ASP; §§ $p < 0.01$, §§§ $p < 0.001$ vs. GLU.

3.4. Proteins Involved in Mitochondrial Oxidative Metabolism Are Differentially Affected by Glucose and Fructose Feeding

To determine whether caloric and non-caloric sweeteners might influence mitochondrial fatty acid oxidation and glucose catabolism downstream of glycolysis, i.e., via the TCA cycle, we quantified the levels of 47 mitochondrial proteins involved in fatty acid β-oxidation, the TCA cycle, and the oxidative phosphorylation pathway in total liver protein extracts. In agreement with the PPARα protein expression pattern, a number of enzymes involved in mitochondrial fatty acid β-oxidation were downregulated by glucose but not by other sweeteners. These enzymes include enoyl-CoA hydratase (ECHS1), electron transfer flavoprotein (subunit ETFB), electron transfer flavoprotein-ubiquinone oxidoreductase (ETFDH), and the liver isoform of carnitine palmitoyltransferase 1 (CPT1A) (Figure 6A). The downregulation of these enzymes suggests a degree of suppression of fatty acid oxidation by glucose. However, increased protein concentrations of trifunctional protein subunits HADHA and HADHB may indicate a compensatory response. Fructose and aspartame largely had no effect on protein concentrations of β-oxidation enzymes, except for downregulation of CPT1A by fructose and trifunctional protein subunit HADHA by aspartame, and upregulation of ECHS1 by aspartame (Figure 6A and Table A1).

The proteomics of TCA cycle enzymes showed that both glucose and fructose caused an increase in protein levels of two components of the acetyl-CoA producing pyruvate dehydrogenase complex, i.e., pyruvate dehydrogenase E1 component subunit α (PDHA1) and dihydrolipoyllysine-residue acetyltransferase (E2 component; DLAT), and the effect was more profound in the FRUC group compared with the GLU group (Figure 6B). The protein concentration of the citrate transporter (SLC25A1) was only affected by glucose feeding, but a number of TCA cycle enzymes downstream of citrate were downregulated by both glucose and fructose (Figure 6B), suggesting a negative regulation of TCA cycle activity by both sweeteners. Aspartame feeding largely had no effect on concentrations of TCA cycle enzymes except for downregulation of 2-oxoglutarate dehydrogenase (OGDH) (Figure 6B and Table A1).

The analysis of enzymes involved in the oxidative phosphorylation pathway showed that this pathway was largely unaffected by the sweeteners (Table A1), except for downregulation of phosphate carrier protein (SLC25A3) and adenine nucleotide translocase 2 (SLC25A5) (Figure 6C), indicating decreased supply of substrates for mitochondrial ATP synthesis.

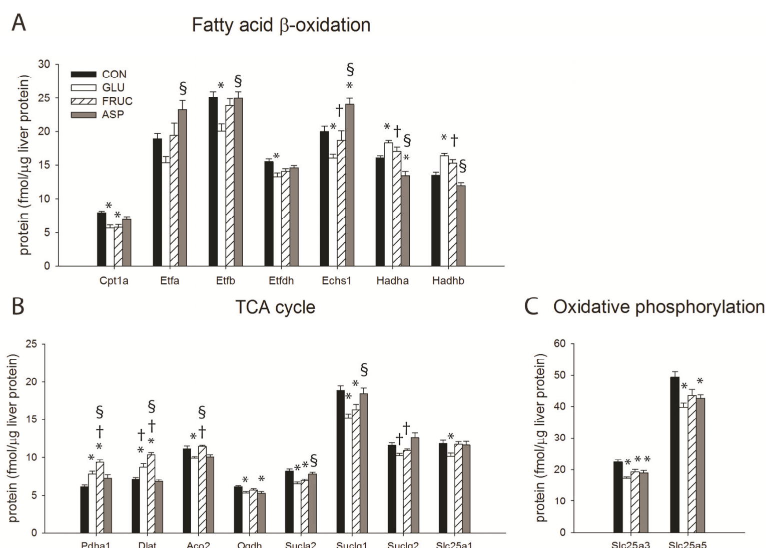

Figure 6. Targeted quantitative mitochondrial proteomics of proteins involved in (**A**) fatty acid β-oxidation; (**B**) tricarboxylic acid (TCA) cycle; and (**C**) oxidative phosphorylation, in livers of rats receiving normal water (CON), a 13% (*w/v*) glucose solution (GLU), a 13% (*w/v*) fructose solution (FRUC), or a 0.4% (*w/v*) aspartame solution ($n = 6$ per diet group). Gene names and corresponding protein names: Cpt1a: Carnitine O-palmitoyltransferase 1A; Etfa: Electron-transfer-flavoprotein, alpha polypeptide; Etfb: Electron-transfer-flavoprotein, beta polypeptide; Etfdh: Electron-transferring-flavoprotein dehydrogenase; Echs1: Enoyl CoA hydratase, short chain, 1; Hadha: Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit; Hadhb: Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit; Pdha1: Pyruvate dehydrogenase E1 component subunit alpha; Dlat: Dihydrolipoamide S-acetyltransferase; Aco2: Aconitase 2; Ogdh: Oxoglutarate (alpha-ketoglutarate) dehydrogenase; Sucla2: Succinyl-CoA ligase [ADP-forming] subunit beta; Suclg1: Succinate-CoA ligase, alpha subunit; Suclg2: Succinate-CoA ligase, beta subunit; Slc25a1: Solute carrier family 25, member 1; Slc25a3: Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; Slc25a5: Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 5. Data are expressed as means ± SEM. * $p < 0.05$ vs. CON; † $p < 0.05$ vs. ASP; § $p < 0.05$ vs. GLU.

4. Discussion

In the present study, we aimed to elucidate the effects of caloric and non-caloric sweeteners on in vivo liver lipid metabolism in rats and to determine their roles in the development of liver steatosis. We showed that fructose and not glucose consumption led to an increase in hepatic lipid content, which was accompanied by an increased conversion of ^{13}C-labeled glucose to lipids in the liver as determined in vivo by MRS. Therefore, de novo lipogenesis appears to be an important contributor to fructose-induced liver lipid accumulation, which was confirmed by the strongly increased expression of lipogenic enzymes upon fructose feeding. Aspartame consumption did not significantly affect hepatic lipid content or metabolism, while it similarly reduced whole-body glucose homeostasis compared with glucose and fructose.

The rise in obesity and obesity-related disorders has been associated with the increased consumption of fructose over the past decades [6,7]. In Western diets, fructose is mainly consumed through sugar-sweetened beverages, containing 10–13% (*w*/*v*) of fructose [39]. In rat models, the feeding of fructose both in pelleted diets and in the drinking water has been shown to lead to hypertriglyceridemia [18,40]. However, in contrast to high-fructose pelleted diets that contain 50–70% fructose, diets incorporating 10% (*w*/*v*) of fructose in drinking water have been reported not to modify plasma glucose or insulin concentrations [18]. In the current study, we therefore chose to use 13% (*w*/*v*) of fructose or glucose in the drinking water, which is also better comparable with the consumption of sugar-sweetened beverages by humans. We observed that fructose feeding resulted in significantly elevated plasma TG concentrations when compared with aspartame feeding, whereas this effect was not observed with glucose feeding. Plasma glucose concentrations were not significantly affected, but plasma insulin levels tended to be increased upon the feeding of both glucose and fructose. Therefore, effects of hyperinsulinemia on liver lipid metabolism cannot be excluded, but these effects are expected to be similar for glucose and fructose feeding.

The animals that received the sugar-sweetened drinking water consumed up to 50% less pelleted chow than the animals receiving normal drinking water or the aspartame solution. Nevertheless, the total energy intake in the sugar groups was higher, due to the calories from the sugar in their drinking water. The increased total energy intake in both sugar groups did, however, not lead to a greater gain in body weight compared with the animals receiving normal drinking water or the aspartame solution. This is in agreement with previous studies administering 10% fructose and/or glucose solutions to rats [18,41–43] and may be explained by the lower dietary quality of their diets (less protein, micronutrients, fiber, and trace elements) as a result of a lower intake of pelleted chow. Because body weight is not always a good indicator of adiposity, especially in animals, analysis of the body composition by determination of fat pad mass is preferred [44]. In the current study, epididymal and perirenal fat pad weights were higher in the two sugar groups compared with the control and aspartame groups, showing that despite the absence of a significant effect on body weight, glucose and fructose feeding led to increased adiposity.

When comparing between the two sugar groups, the addition of glucose to the drinking water resulted in a higher total energy intake compared with fructose, which was due to a higher consumption of the glucose solution compared with the fructose solution. However, the consumption of pelleted chow was higher in the fructose-fed animals compared with the glucose-fed animals. This observation is in agreement with a previous study in rats, in which the less pronounced reduction in solid food consumption upon fructose feeding was explained by a state of leptin resistance produced by the ingestion of fructose [18]. However, in humans, differential effects of glucose and fructose on ad libitum energy intake have not been observed [45].

Seven weeks of fructose administration resulted in an increased amount of lipids in the liver, whereas the administration of glucose did not significantly affect liver lipid content compared with normal drinking water. This observation is in accordance with previous studies comparing the effects of glucose and fructose on liver lipid accumulation both in rodents [18,46,47] and in humans [48].

In order to determine the cause of liver lipid accumulation upon fructose consumption, ^{1}H-(^{13}C) MRS was performed combined with the oral administration of ^{13}C-labeled lipids, to examine dietary lipid uptake, and with the oral administration of ^{13}C-labeled glucose, to determine de novo lipogenesis. Chronic fructose feeding has been shown to lead to an overproduction of intestinal apoB48-containing lipoproteins, which was associated with greater stability of intracellular apoB48 and upregulation of the key enzyme involved in intestinal lipoprotein assembly, microsomal TG transfer protein [19–21]. Therefore, chronic fructose consumption may promote the absorption of dietary lipids from the intestine, resulting in an increased influx of diet-derived lipids into the liver. However, 4 h after the administration of ^{13}C-labeled lipids, the hepatic incorporation of the ingested dietary lipids was similar among all diet groups. Nunes et al. applied the same MRS-based method to determine dietary lipid uptake in the livers of mice fed with pelleted diets containing 60% glucose or fructose for a period of 8 weeks [47]. Also in this study, the liver lipid pools became equally ^{13}C enriched in the glucose- and fructose-fed animals after the ingestion of ^{13}C-labeled lipids, but no control group fed with normal chow was included. It was furthermore demonstrated that plasma concentrations of apoB48 and apoB100 were similar between the glucose- and fructose fed groups [47]. Together, these results do not support the hypothesis that an increased influx of dietary lipids into the liver causes fructose-induced hepatic lipid accumulation.

We then investigated the contribution of de novo lipogenesis to hepatic lipid accumulation by administering the rats with ^{13}C-labeled glucose for a period of 5 days, after which the conversion of ^{13}C-labeled glucose to ^{13}C-labeled lipids in the liver was measured with in vivo ^{1}H-[^{13}C] MRS. We showed that the ^{13}C enrichment of the liver lipid pool upon ^{13}C-labeled glucose administration was 3.5-fold higher in fructose-fed animals compared with animals receiving normal drinking water, and, moreover, that it was 2.8-fold higher in fructose-compared with glucose-fed animals. Therefore, de novo lipogenesis appears to be an important contributor to fructose-induced liver lipid accumulation.

The stimulation of de novo lipogenesis upon fructose feeding was further investigated by determining the protein levels of lipogenic enzymes, ACC and FAS, and of transcription factors involved in the transcriptional regulation of lipogenic genes, SREBP-1c and ChREBP. Glucose and fructose feeding similarly increased the expression of ChREBP (only significantly for glucose versus normal water) and precursor SREBP-1c in the liver, but the protein levels of mature SREBP-1c were not elevated. However, despite the absence of a clear stimulating effect of fructose on ChREBP and SREBP-1c with respect to glucose, the expression levels of ACC and FAS were 1.6-fold and 1.5-fold higher in fructose- compared with glucose-fed animals, respectively. These results confirm the findings of previous studies using sugar-sweetened drinking water [15,18,49]. A possible explanation for the increased protein levels of ACC and FAS in fructose- compared with glucose-fed animals without differences in ChREBP and SREBP-1c could be that the nuclear fractions of ChREBP and SREBP-1c are responsible for inducing the expression of these lipogenic enzymes [13,14], whereas in the current and other studies, protein levels were determined in whole liver homogenates. Janevski et al. [14] showed that in rats fed with diets containing 60% glucose or 60% fructose, protein levels of ChREBP and SREBP-1c were similar in liver homogenates, but were higher in the nuclear fractions from livers of fructose-fed animals, and that this was associated with increased ACC and FAS gene transcription.

Mitochondrial proteomics provided additional proof for the fructose-induced stimulation of de novo lipogenesis. In livers of both glucose- and fructose-fed animals, two components of the pyruvate dehydrogenase complex were upregulated (PDHA1, DLAT), suggesting increased production of acetyl-CoA [40]. This was in agreement with increased activities of glycolytic enzymes, in particular pyruvate kinase, a phenomenon also reported by others [50,51]. Decreased protein levels of several TCA cycle enzymes (ACO2, OGDH, SUCLA2, and SUCLG1), all of them catalyzing reactions downstream of citrate, suggest that TCA cycle activity might have been limited upon glucose and fructose feeding, favoring the translocation of citrate to the cytosol for lipogenesis [52]. Importantly, most of these effects were more profound in response to fructose compared with glucose.

Decreased mitochondrial fatty acid β-oxidation capacity may contribute to the accumulation of hepatic lipids. The protein levels of transcription factor PPARα, which regulates fatty acid catabolism, was decreased in response to glucose but not fructose feeding. However, Roglans et al. showed that fructose feeding reduces PPARα activity without modifying hepatic PPARα protein levels [18]. In agreement, a downregulation of CPT1A, a rate limiting enzyme in mitochondrial β-oxidation, was observed in livers of both glucose- and fructose-fed animals, indicating decreased mitochondrial acyl-CoA uptake and possibly β-oxidation. However, only glucose also induced upregulation of mitochondrial trifunctional protein (subunits HADHA and HADHB), possibly indicating a compensatory response resulting in lesser suppression of β-oxidation compared to fructose. Furthermore, in the livers of fructose-fed animals, we observed accumulation of the CPT1A inhibitor malonyl-CoA, leading to stronger inhibition of fatty acid β-oxidation compared to glucose [15]. The shift of fatty acids away from oxidation and towards esterification will cause an increase in very-low density lipoprotein (VLDL) secretion, which can explain the increased plasma TG levels in the fructose-fed animals [7,9,53,54]. However, taken together our data suggest that hepatic lipid export was not matched to increased production and decreased oxidation.

The administration of aspartame for 7 weeks did not have a significant effect on food intake or body weight compared with controls receiving normal drinking water. The absence of an effect on body weight is in agreement with a study conducted by Palmnas et al., in which rats received a low dose of aspartame in their ad libitum drinking water (5–7 mg/kg/day) for 8 weeks [31]. Those animals, however, consumed less food and drank more fluid compared to the control animals, which was not observed in the current study. Low dose aspartame consumption in drinking water has been shown to increase fasting blood glucose levels and induce glucose intolerance without an effect on fasting plasma insulin levels in both rats [31] and mice [26]. The effects were attributed to compositional and functional changes in gut microbiota, resulting in increased production of short-chain fatty acids. Short-chain fatty acids can serve as gluconeogenic precursors, possibly contributing to increased hepatic glucose production and thus explaining aspartame's negative effects on glucose tolerance. In a recent study, however, it was shown that inhibition of the gut enzyme intestinal alkaline phosphatase by aspartame's breakdown product phenylalanine may explain how aspartame promotes glucose intolerance [30]. In the present study, rats consumed a higher dose of aspartame (280 mg/kg/day) compared to the low dose of 5–7 mg/kg/day reported in [31] and the dose of 123.3 mg/kg/day used in [30]. In contrast to those studies, we did not observe an effect of aspartame on blood glucose levels, but aspartame feeding led to higher fasting plasma insulin levels and trends in higher AUC_i and the product of AUC_g and AUC_i compared with controls. This suggests that higher doses of aspartame may induce compensatory insulin production in the pancreas, resulting in maintenance of normal fasting blood glucose levels and glucose disposal.

To date, the effects of aspartame on liver lipid metabolism have been largely unknown. Exploring the impact of aspartame consumption on hepatic dietary lipid uptake and de novo lipogenesis with ^{1}H-[^{13}C] MRS revealed no significant effects and total liver lipid content was similar between animals receiving aspartame or normal drinking water. The expression of most of the transcription factors and enzymes involved in lipid metabolism was also not significantly affected by aspartame feeding, corroborating that liver lipid metabolism is largely unaffected by aspartame. Despite of this, aspartame feeding similarly reduced whole-body glucose homeostasis compared with glucose and fructose feeding. In the case of glucose and especially fructose, derangements in liver lipid metabolism are thought to contribute to the development of hepatic insulin resistance [11]. Our results indicate that changes in liver lipid content or metabolism do not play a causative role in aspartame-induced glucose intolerance.

5. Conclusions

In conclusion, both glucose and fructose feeding increased adiposity, but only fructose led to higher hepatic lipid levels. The increase in hepatic lipid content upon fructose consumption was not caused by an increased uptake of dietary lipids into the liver, but could be attributed to an increase in de novo lipogenesis and presumably a reduction in fatty acid β-oxidation. The non-caloric sweetener aspartame did not significantly affect liver lipid content or metabolism, while its effects on whole-body glucose homeostasis were comparable to glucose and fructose.

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

Appendix A. Experimental Procedures

Western Blot Analysis

Powdered liver samples were homogenized in ice cold lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris, 2 mM NaF, 2 mM Na_3VO_4, protease inhibitor cocktail (1:200), pH 8. Homogenates were solubilized for 2 h at 4 °C and centrifuged at 14,000× g for 10 min at 4 °C. Equal amounts (30 µg) of total liver protein were resolved with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using Trans-Blot Turbo Midi Nitrocellulose Transfer Packs, and Trans-Blot Turbo Transfer Starter System (Bio-Rad Laboratories Inc., Hercules, CA, USA). After blocking with Tris-buffered saline (TBS) containing 0.1% Tween (TBST) and 5% skim milk powder for 1 h at room temperature, the membranes were incubated overnight at 4 °C with antibodies against acetyl-CoA carboxylase (ACC, 1:1000, cat. No. 3662, Cell Signaling, Danvers, MA, USA), fatty acid synthase (FAS, 1:1000, cat. No. 3180, Cell Signaling), sterol regulatory element-binding protein-1c (SREBP-1c) precursor and cleaved protein (1:1000, cat. No. NB600-582, Novus Biologicals, Littleton, CO, USA), carbohydrate-responsive element-binding protein (ChREBP, 1:1000, cat. No. NB400-135, Novus Biologicals), peroxisome proliferator-activated receptor α (PPARα, 1:1000, cat. No. sc-9000, Santa Cruz Biotechnology), and β-actin (1:5000, cat. No. 2066, Sigma-Aldrich). Next, membranes were washed 3 × 5 min with TBST and incubated with a corresponding horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature. After the final wash of 3 × 5 min with TBST and 1 × 5 min with TBS, the immunocomplexes were detected using SuperSignal West Dura Extended Duration Substrate (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA), visualized using ChemiDoc XRS+ imaging system, and quantified using Image Lab analysis software version 3.0 (Bio-Rad Laboratories Inc., Hercules, CA, USA). All data were normalized to β-actin protein levels and expressed relative to the controls (CON).

Table A1. Targeted quantitative mitochondrial proteomics in liver.

Gene Name	Protein Name	CON	GLU	FRUC	ASP
	Fatty acid β-oxidation				
Acaa2	Acetyl-CoA acyltransferase 2	34.2 ± 0.7	32.5 ± 1.2 †	36.5 ± 2.3	39.0 ± 1.7
Acadl	Acyl-CoA dehydrogenase, long-chain	38.7 ± 1.3	34.9 ± 1.6	39.0 ± 2.1	41.0 ± 1.9
Acads	Acyl-CoA dehydrogenase, short-chain	11.4 ± 0.5	10.5 ± 0.2	10.9 ± 0.3	10.1 ± 0.4
Acadvl	Acyl-CoA dehydrogenase, very long-chain	12.5 ± 0.6	11.5 ± 0.2	10.1 ± 0.6 *	11.0 ± 0.5
Cpt1a	Carnitine O-palmitoyltransferase 1A	8.0 ± 0.2	5.7 ± 0.4 **	5.8 ± 0.4 **	7.0 ± 0.4
Cpt1b	Carnitine O-palmitoyltransferase 1B	1.29 ± 0.07	1.96 ± 0.19	1.46 ± 0.12	1.65 ± 0.38
Cpt2	Carnitine palmitoyltransferase 2	6.2 ± 0.2	6.8 ± 0.3	6.5 ± 0.6	5.4 ± 0.3
Decr1	2,4-dienoyl CoA reductase 1	7.8 ± 0.22	7.4 ± 0.3 ††	8.3 ± 0.7 †	10.0 ± 0.2 **
Echs1	Enoyl CoA hydratase, short chain, 1	20.0 ± 0.8	16.0 ± 0.7 *,†††	18.7 ± 1.5 ††	24.1 ± 0.9 *
Eci1	Enoyl-CoA delta isomerase	11.9 ± 0.7	11.8 ± 0.7	12.0 ± 1.0	13.1 ± 0.6
Etfa	Electron-transfer-flavoprotein, alpha polypeptide	18.9 ± 0.8	15.4 ± 1.0 ††	19.5 ± 1.8	23.3 ± 1.3
Etfb	Electron-transfer-flavoprotein, beta polypeptide	25.1 ± 0.8	20.1 ± 1.0 **,††	23.9 ± 1.0	25.0 ± 1.0
Etfdh	Electron-transferring-flavoprotein dehydrogenase	15.5 ± 0.4	13.3 ± 0.6 **	14.1 ± 0.4	14.6 ± 0.3
Hadh	Hydroxyacyl-CoA dehydrogenase	34.4 ± 1.1	30.9 ± 1.2	32.7 ± 1.2	34.0 ± 1.2
Hadha	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	16.1 ± 0.3	18.3 ± 0.4 *,†††	17.1 ± 0.6 †††	13.5 ± 0.6 **
Hadhb	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	13.6 ± 0.4	16.5 ± 0.3 **,†††	15.3 ± 0.5 †††	12.0 ± 0.4
Slc25a20	Solute carrier family 25, member 20	4.1 ± 0.2	3.1 ± 0.3 †††	4.4 ± 0.3 §	5.4 ± 0.4 *
	TCA cycle				
Aco2	Aconitase 2	11.1 ± 0.4	9.9 ± 0.2 *	11.4 ± 0.1 §§,†	10.1 ± 0.3
Cs	Citrate synthase	5.40 ± 0.13	4.66 ± 0.14 ††	5.59 ± 0.39 §	6.04 ± 0.09
Dlat	Dihydrolipoamide S-acetyltransferase	7.1 ± 0.2	8.7 ± 0.5 *,††	10.3 ± 0.3 ***,§§,†††	6.8 ± 0.2
Dld	Dihydrolipoamide dehydrogenase	23.3 ± 0.7	21.2 ± 1.1	23.3 ± 1.1	20.7 ± 0.4
Dlst	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	13.4 ± 0.8	11.7 ± 0.6	13.2 ± 0.2	13.3 ± 0.4
Fh1	Fumarate hydratase 1	14.1 ± 0.7	12.1 ± 0.6	15.9 ± 1.5	16.0 ± 1.0
Idh2	Isocitrate dehydrogenase 2 (NADP+)	10.0 ± 0.4	9.2 ± 0.6	9.2 ± 0.2	9.0 ± 0.3

Table A1. *Cont.*

Gene Name	Protein Name	CON			GLU			FRUC			ASP		
Idh3a	Isocitrate dehydrogenase [NAD] subunit α	3.3	±	0.4	3.1	±	0.5	2.7	±	0.2	3.2	±	0.2
Mdh2	Malate dehydrogenase 2	31.6	±	1.0	27.2	±	0.9 †	31.2	±	2.0	33.8	±	1.6
Ogdh	Oxoglutarate (alpha-ketoglutarate) dehydrogenase	6.1	±	0.1	5.3	±	0.2 *	5.7	±	0.2	5.3	±	0.2 *
Pdha1	Pyruvate dehydrogenase E1 component subunit alpha	6.1	±	0.2	7.8	±	0.4 *	9.4	±	0.3 ***,§,††	7.3	±	0.5
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1	1.06	±	0.01	1.06	±	0.03 ††	1.17	±	0.02 **,††	1.05	±	0.02
Slc25a1	Solute carrier family 25, member 1	11.8	±	0.4	10.2	±	0.4 *	11.7	±	0.3	11.6	±	0.5
Slc25a10	Solute carrier family 25, member 10	9.6	±	0.5	6.8	±	0.5 **,††	8.0	±	0.4	9.5	±	0.6
Slc25a11	Solute carrier family 25, member 11	5.5	±	0.4	4.6	±	0.2	4.7	±	0.4	5.1	±	0.3
Slc25a22	Solute carrier family 25, member 22	3.99	±	0.11	3.31	±	0.09 **,†	3.22	±	0.11 **,††	3.85	±	0.15
Sucla2	Succinyl-CoA ligase [ADP-forming] subunit beta	8.2	±	0.3	6.5	±	0.2 ***,††	6.9	±	0.2 **	7.8	±	0.3
Suclg1	Succinate-CoA ligase, alpha subunit	18.9	±	0.6	15.2	±	0.5 **,†	16.3	±	0.7 *	18.5	±	0.7
Suclg2	Succinate-CoA ligase, beta subunit	11.6	±	0.3	10.3	±	0.3 ††	10.9	±	0.2 †	12.7	±	0.6
	Oxidative phosphorylation												
Ndufs1	NADH dehydrogenase (ubiquinone) Fe-S protein 1	5.4	±	0.3	4.5	±	0.2 *	5.8	±	0.2 §§	5.1	±	0.2
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein	10.0	±	0.3	8.9	±	0.3 *	9.7	±	0.2	9.9	±	0.3
Sdhb	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	6.8	±	0.2	5.1	±	0.1 *,†††	6.9	±	0.5 §§	7.4	±	0.3
Uqcrc2	Ubiquinol-cytochrome c reductase core protein II	8.5	±	0.3	7.3	±	0.3 †††	9.1	±	0.5 §	9.9	±	0.4
Cox5a	Cytochrome c oxidase subunit Va	5.31	±	0.24	4.86	±	0.23 ††	5.70	±	0.08 §	5.79	±	0.11
Cycs	Cytochrome c, somatic	3.26	±	0.25	2.94	±	0.14	2.88	±	0.09	3.13	±	0.13
Atp5b	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	63.2	±	2.1	58.4	±	2.5 †	71.5	±	5.2 §	73.0	±	2.1
Slc25a3	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	22.4	±	0.7	17.2	±	0.5 ***	19.3	±	0.9 *	19.0	±	0.8 *
Slc25a4	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 4	0.97	±	0.04	0.87	±	0.05	0.86	±	0.02	0.83	±	0.03
Slc25a5	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 5	49.4	±	1.7	39.8	±	1.3 **	43.6	±	1.9	42.7	±	1.1 *
Ucp2	Uncoupling protein 2	0.80	±	0.04	0.84	±	0.06	0.70	±	0.04	0.71	±	0.06
Ucp3	Uncoupling protein 3	0.92	±	0.04	1.08	±	0.12 †	0.81	±	0.04	0.78	±	0.05

Data are expressed as means ± SEM of six animals per diet group. CON, normal water control; GLU, 13% (w/v) glucose; FRUC, 13% (w/v) fructose; ASP, 0.4% (w/v) aspartame. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CON; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. ASP; § $p < 0.05$, §§ $p < 0.01$ vs. GLU.

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