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The Effect of Fructose vs. Glucose Beverages on
Low-Grade Systemic Inflammation

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Abstract

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The epidemics of obesity and related metabolic diseases are a serious health concern worldwide. These conditions are multifactorial in origin and develop over time; however one factor that continues to be implicated in the development of obesity is the intake of sugar-sweetened beverages (SSBs). Consumption of SSBs is also associated with type 2 diabetes (T2DM) and cardiovascular disease (CVD), both independently and through the increased risk conferred by excess body fat. It is not entirely clear though which mechanism(s) SSB consumption might contribute to excess body weight and an increased risk of T2DM and CVD. However, low-grade chronic inflammation underlies both of these diseases and might be a potential link between the SSB-metabolic disease association. It is also unclear whether it is the fructose, rather than the glucose, component of the sweetener that might be responsible for the ill effects associated with SSB consumption. To address these questions, we conducted a randomized, controlled, double-

blind crossover design dietary intervention trial to test the effects of beverages sweetened with fructose, high fructose corn syrup (55% fructose, 45% glucose), or glucose on total energy intake and low-grade chronic inflammation. We recruited 12 overweight or obese and 12 normal weight men and women who were free of chronic inflammatory or metabolic disease. Each subject completed each of three 8-day dietary periods during which they consumed four servings per day of beverages sweetened with either fructose, HFCS, or glucose in addition to standardized solid foods that were consumed *ad libitum*. Total energy intake during the fructose, HFCS, and glucose phases was $116\% \pm 14\%$, $116\% \pm 16\%$, and $116\% \pm 16\%$ of the subject's estimated total energy requirements ($p=0.880$). In terms of inflammation, fasting plasma concentrations of C-Reactive Protein (CRP) ($p=0.457$) and interleukin-6 ($p=0.933$) did not differ to a statistically significant degree at the end of the 3 diet periods. Nor did we detect a consistent differential effect of the diets on measures of adipose tissue inflammation. However, adiponectin gene expression in adipose tissue ($p=0.005$) was statistically significantly lower following the glucose and HFCS phases compared to the fructose phase. We also did not detect consistent evidence of a differential impact of fructose vs. glucose on measures of intestinal permeability (lactulose-mannitol test, plasma zonulin, and plasma lipopolysaccharide-binding protein). Our results indicate that in normal weight to obese adults, consumption of SSBs promotes an increase in overall energy intake over 8 days, which would be expected to result in weight gain over the longer term. Because total energy intake was elevated to the same degree above subjects' estimated energy requirements, we conclude that we found no evidence that fructose, HFCS, and glucose differ in terms of their ability to regulate energy intake when consumed in the form of an SSB. Instead, it appears that these sugars consumed in liquid form fail to adequately reduce energy intake from solid foods thereby leading to weight gain. Furthermore, despite this increase

in total energy intake, we observed no evidence that consumption of these SSBs differentially affected markers of systemic inflammation, adipose tissue inflammation, or intestinal permeability. Taken together, the increased risk for cardiometabolic disease associated with SSB intake is most likely mediated by adiposity which is the result of excess energy intake over time promoted by incomplete compensation for calories from sugars consumed in liquid form.

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1. BACKGROUND AND SIGNIFICANCE

1.1. The twin epidemics of obesity and type 2 diabetes as a major public health challenge

From a global perspective, there are few public health issues in history that have affected the population to the same extent as the epidemics of obesity and type 2 diabetes mellitus (T2DM) (1, 2). As of 2012, 35% of adults in the US were obese (BMI > 30 kg/m²) (3); which predisposes individuals to T2DM at a rate 7 times higher than that for normal weight individuals (4). Yet, the mechanisms and sequence of events initiated by the accumulation of excess body fat and culminating in outright T2DM have not been conclusively defined. Furthermore, there is no body fat threshold above which T2DM develops, and the etiology is thought to be multifactorial. Because T2DM alone is responsible for an estimated \$245 billion in direct medical costs and lost productivity annually (5), research in this area is a priority of the US government and the National Institutes of Health.

From a historical and evolutionary perspective, obesity and diabetes are predominantly diseases of lifestyle rather than of genetic origin (6). Therefore, much effort has been devoted to determining modifiable environmental and lifestyle factors that contribute to their etiology. Large, population-based observational studies have been useful in identifying factors that are associated with these diseases. One dietary component that continues to be implicated in the development of obesity is the intake of sugar-sweetened beverages (SSBs) (7). Sugar-sweetened beverages are a major source of added sugars in the US diet and include any beverages sweetened with added sugars, including soft drinks, fruit drinks (excluding 100% juice), sweetened coffee and tea, energy drinks, and flavored waters. These beverages account for almost half of all added sugars consumed by the US population (8) and are usually sweetened with a combination of glucose and fructose in the form of high-fructose corn syrup (HFCS) or sucrose. The most commonly used formulation of HFCS contains 55% fructose, 41% glucose, and 4% higher saccharides; however, a recent survey of SSBs commonly consumed in the US indicated that the fructose content varies widely and is often as high as 75% fructose (9). In 2014, average US per capita intake of caloric sweeteners, including HFCS and sucrose, was 23 teaspoons (365 kcals) per day (10).

1.2. Sugar-sweetened beverage intake is associated with obesity and cardiometabolic disease

1.2.1. Sugar-sweetened beverage intake and adiposity

A large body of observational studies has consistently reported a positive, linear association between SSB intake and weight gain or risk of obesity (11-13). Based on estimates from the most recent cohort studies, a 1-serving/day increase in SSB intake was associated with an additional average weight gain of 0.12 kg per year (14). Given that adults in the US gain 0.5 to 1 kg each year on average during adulthood (15), a 0.12 kg per year increase in body weight attributable solely to each serving/d of SSB consumed is substantial. Evidence from intervention studies also shows that the addition of SSBs to the diet statistically significantly increases body weight (12) in a dose-dependent manner (16).

Results from the Framingham's Third Generation cohort also indicate that greater SSB intake is associated with an increase in fat mass. Specifically, abdominal adipose tissue was measured in over 1,000 participants in the cohort at two clinic visits separated by six years. At baseline and follow-up, SSB and artificially sweetened-beverage intakes were assessed by food frequency questionnaire (FFQ). At follow-up, higher SSB intake was associated with a statistically significantly greater change in visceral adipose tissue (VAT) compared to consumption of non-calorically sweetened beverages (17).

The strongest evidence of the contribution of SSBs to weight gain comes from a well-controlled, double blind trial in children and adolescents carried out by de Ruyter et al. (18). Investigators randomized 641 normal weight children to receive 8 oz/d of either an SSB or artificially sweetened beverage for 18 months. At follow-up, the SSB group had gained statistically significantly more weight (16%) and body fat compared to the control group, after adjusting for the normal growth curve in children. This result is striking in that a statistically significant effect was seen even with a fairly low SSB dose. In the US, children consume, on average, almost three times the calories from SSBs as was provided in this trial (18). Similarly, Ebbeling et al. (19) conducted a randomized intervention designed to reduce SSB intake in overweight and obese adolescents. After 1 year, the group that received the intervention successfully reduced their SSB intake and their increase in BMI was statistically significantly smaller compared to the control group who received no intervention (19). The effect had diminished by the end of the second year, during which subjects did not receive contact with study staff. It should be noted that the

control group did not receive the equivalent amount of intervention materials or contact hours with study staff during the first year compared to the intervention group. However, because the weight-loss effect did not persist into the second year, when there were higher rates of recidivism in SSB consumption in the intervention group, the hypothesis that decreasing SSB intake does indeed translate into meaningful changes in body weight was further strengthened. Some assert that the strength of the outcomes from these combined studies indicate a conclusive, causal link between SSB consumption and obesity (20).

While perhaps too early to assess hard outcomes, many countries and municipalities are in the process of implementing a tax on SSBs in an attempt to curb the incidence of obesity. On a larger population scale, this will provide further evidence of any association between SSB consumption and obesity that might exist, assuming that consumption is reduced in response to an increased cost of SSBs. While the longer term public health impact of enacting such a tax has yet to be determined, early evidence suggests that taxation is an effective strategy to decrease SSB consumption (21).

In summary, the overwhelming majority of evidence supports the statement that SSBs promote weight gain and adiposity in children and adults, and reducing SSB consumption would likely result in beneficial effects on body weight (20).

1.2.2. Sugar-sweetened beverage intake and type 2 diabetes mellitus

Evidence from observational studies also indicates a consistently strong, positive association between SSB intake and an increased risk of developing T2DM (13, 20, 22). In most prospective cohort studies, weight gain occurred concurrently with a decline in metabolic health, which indicates that adiposity is a potential mediator of the association between SSB consumption and T2DM. However, after adjustment for BMI and energy intake, the association, although attenuated, still remained statistically significant in most cases suggesting that SSB consumption may contribute to the etiology of T2DM through metabolic pathways partially independent of adiposity (14).

The majority of cohort studies on this topic have been grouped and summarized in several meta-analyses. The consensus from these analyses has been that those in the highest category of SSB intake (1 to 2 servings/d) had a 26-30% greater risk of developing diabetes compared to those

with the lowest intake (< 1 serving/month) (22, 23). Or put another way, a 20% increase in risk of T2DM per 330 mL SSB consumed per day (24). Across the board, the association between SSB intake and risk of T2DM was attenuated, but remained statistically significant, after adjusting for BMI and energy intake which suggests that the link between SSB intake and T2DM is not completely mediated by adiposity. Overall, the combined data indicate that on average, a 1-serving per day increase in SSB consumption is associated with a 20-25% increased risk of T2DM, and a 13% greater risk after adjusting for BMI (14).

Differences in the relative risk estimates determined from the different cohort studies can be partially attributed to the manner in which each study defined ‘sugar-sweetened beverages’. For instance, some studies included all ‘carbonated drinks’ as SSBs, which also captured artificially sweetened-beverage intake. Furthermore, the term ‘juice’ often includes both 100% fruit juice in addition to fruit-flavored drinks. Consensus does not exist about whether 100% juice should be included in the SSB category, and therefore is left to the discretion of the authors of the individual studies. These differences notwithstanding, the overwhelming body of observational studies supports the positive association between SSB intake and increased risk of T2DM (25).

Observational studies measuring biomarkers of T2DM risk, such as HOMA-IR (homeostatic model assessment of insulin resistance), insulin sensitivity, and beta-cell function as endpoints can offer a more short-term assessment of SSB intake and prediction of incident T2DM. One such study included 546 European adolescents and assessed SSB intake by FFQ and HOMA-IR by fasting blood sample. While this was a cross-sectional analysis, it was the first large study that specifically examined the association between SSB intake and insulin resistance (26). In this cohort of 12-18 yo, mean HOMA-IR values were statistically significantly higher in those consuming SSBs > 5x/wk compared to < 4x/wk. After controlling for BMI, total energy intake, and physical activity, regression analysis indicated that those consuming SSB at least 5x/wk had statistically significantly higher HOMA-IR (by 0.281 units) compared to those consuming SSBs less than 1x/wk (26). This finding is in line with evidence from other observational studies and suggests that daily SSB consumption is strongly associated with elevated HOMA-IR independent of body weight.

Evidence of an adiposity-independent association between SSB intake and T2DM risk was provided by a cross-sectional study carried out in over 7,800 adults living in Spain. A 1-

servings/d (200 mL/d) increase in SSB consumption was associated with 2-fold higher plasma insulin and HOMA-IR and 2.7-fold higher leptin in men, after adjustment for BMI, waist circumference, and morbidity. In non-overweight women, the same consumption was associated with 3-fold higher insulin and HOMA-IR and 4.6-fold higher leptin, after adjustment for confounders and potential mediators (27). This study indicates that early signs of metabolic dysfunction are associated with SSB intake and that the association between SSB consumption and risk factors for T2DM is not entirely dependent on adiposity, given that the association was seen even in non-overweight women, and even after adjustment for measures of adiposity.

Similarly, in a group of 63 overweight Latino children (age 9-13 y), a frequently sampled intravenous-glucose-tolerance test (FS-IVGTT) was performed to assess insulin sensitivity, acute insulin response, and disposition index in relation to SSB intake (28). Habitual diet was assessed by 3-d food records and indicated that over 40% of the carbohydrate intake in this cohort was derived from high sugar foods and, of this, 50% came from SSBs (approximately 550 mL/d). Upon analysis, total sugar intake was statistically significantly associated with a lower acute insulin response to glucose (AIR) and disposition index (DI) independent of body composition and total energy intake (28). This indicates early signs of impaired β -cell function in those Latino children who were habitual SSB consumers, which predicts a substantial increase in their risk of developing T2DM in the future (29).

Experimental evidence from randomized controlled trials (RCTs) on SSB intake and biomarkers of T2DM risk are few due to feasibility, cost, and ethical considerations associated with these interventions. One RCT comparing the effect of daily consumption of SSBs vs. low-fat dairy on insulin sensitivity and pancreatic β -cell function in adults at risk of T2DM who habitually consumed SSBs (30). The trial was a randomized, crossover design and included 34 men and women who were overweight/obese and nondiabetic but who had impaired fasting glucose or elevated hemoglobin A1c (5.7-6.4%) and consumed > 2 servings of SSB/d. The intervention included two, 6-wk periods: A SSB phase, consisting of 710 mL/d of SSB and 108 g/d of a sugar-sweetened, nondairy pudding, and a dairy phase consisting of 474 mL/d of 2% milk and 170 g/d low-fat yogurt with no added sugar, in addition to their habitual diet. The order the subjects received the dairy vs. the SSB phase was randomized and there was a 2-wk washout period between phases. Subjects underwent a liquid meal tolerance test (Ensure beverage) at

baseline and following each intervention phase, and sequential blood draws occurred every 30 min for measurement of plasma glucose and insulin. Overall, insulin sensitivity (HOMA2-%S) decreased by 21% from baseline following the SSB period and increased by 1.3% following the dairy phase, which resulted in a statistically significant difference in the change between diet periods. Disposition index also changed in a less favorable direction following SSB consumption (-0.4), while dairy intake did not influence this biomarker. Fasting insulin also increased to a statistically significant degree from baseline following the SSB phase compared to the dairy phase (a 1.2 vs. - 0.1 uU/mL change, respectively) (30). While β -cell function did not appear to be statistically significantly impacted by the interventions, the decrease in the disposition index and insulin sensitivity suggest that, in this non-diabetic population, β -cell function was not able to compensate for worsening insulin sensitivity. Overall, these data indicate that moderate SSB consumption results in an increase in fasting insulin as well as a decrease in overall insulin sensitivity, which are both indicative of greater risk of T2DM.

Overall, combined evidence from observational studies, including prospective cohorts and cross-sectional studies using biomarkers of T2DM to assess risk, as well as RCTs suggest that even one serving/d (200 mL) of SSBs statistically significantly increases the risk of developing T2DM. The strength of this positive association appears to increase linearly with SSB dose and the risk of T2DM is not mediated by an increase in body weight or adiposity. Furthermore, evidence of impaired insulin sensitivity is apparent in children and adolescents who consume SSBs routinely, and worsening of these endpoints can be induced within just 6 weeks of chronic intake in adults even among those who are already habitual SSB consumers.

1.2.3. Sugar-sweetened beverage intake and cardiovascular disease

While direct evidence of the association between SSBs and cardiovascular disease (CVD) is limited, observational evidence from large cohorts followed over the long term provide some insight. In the NHANES II cohort, added sugar was associated with a 2-fold greater risk of CVD mortality after a median of 14.6 years of follow-up when comparing the lowest to highest quintiles of intake (31). Given that SSBs promote obesity, and obesity predisposes individuals to CVD, the authors aimed to disentangle whether any direct influence of SSBs on CVD risk exists independent of adiposity. Analysis of the Nurses Health Study cohort indicated that those women consuming greater than 2 SSBs per day had a 35% greater risk of coronary heart disease (CHD)

than those who consumed less than 1 SSB per month; and this relationship, although attenuated, remained statistically significant after adjustment for BMI and energy intake (32). This suggests that SSBs contribute to CVD risk partially through weight-gain independent mechanisms.

Due to the cost, feasibility, and length of time required to assess the direct relationship between SSB intake and manifest CVD, intermediate endpoints of CVD are often used to characterize the relationship between exposure and risk of disease. Biomarkers such as blood pressure and blood lipid ratios are frequently used to predict future incident CVD. In a survey of these studies, Malik et al. (13) conducted a systematic review including 10 cohort studies and concluded that there is sufficient evidence supporting a direct association between SSB intake and the development of hypertension, dyslipidemia, inflammation, and clinical CHD. An updated review from the same group, including 12 cohort studies with over 409,000 participants, concluded that SSB intake was directly associated with elevated blood pressure and hypertension, and that intakes of at least 12 oz/d increased the risk of developing hypertension by 6%, after adjustment for adiposity (33). Furthermore, in a meta-analysis including 6 large prospective studies carried out prior to 2014, every additional serving/d in SSB consumption was associated with an 8% and 17% increased risk for incident hypertension and CHD, respectively, after adjustment for adiposity (34).

Analyses from the Health Professionals Study cohort, which included over 42,000 men, found that intake of SSBs was statistically significantly associated with increased plasma triglycerides and leptin: Those individuals in the top quartile of SSB intake had a 20% greater risk of developing CHD compared to those in the bottom quartile of intake after adjustment for BMI and other lifestyle factors (35). Taken together, the available evidence suggests there is a strong association between SSB intake and biomarkers of CVD, which exists even after adjustment for BMI, which indicates that adiposity does not completely mediate the relationship.

Cardiovascular disease is strongly associated with low-grade inflammation, which is now considered to be a causal process underlying development of these chronic diseases (36). However, few observational studies have been conducted that assess the impact of SSB intake on biomarkers of inflammation, such as C-Reactive Protein (CRP) and other plasma cytokines, as they relate to risk of CVD. One study utilizing the Nurses' Health Study and Nurses' Health Study II cohorts concluded that within 'dietary patterns' including high amounts of SSBs, those in the highest quintile of SSB intake had elevated CRP, E-selectin, sICAM-1, sVCAM-1, and

sTNFR2 compared to those in the lowest quintile (37). Inflammatory biomarkers were also measured in the Health Professionals Study cohort within which intake of SSBs was statistically significantly associated with elevated levels of circulating CRP, IL-6, and sTNFR1&2 (35). In both of these studies, the statistically significant association between SSB intake and biomarkers of inflammation remained even after adjustment for BMI, again suggesting that SSB intake might influence CVD risk through mechanisms independent of body weight gain.

Lastly, three RCTs have evaluated the effect of SSB consumption on inflammation and other biomarkers of CVD risk, with discrepant results. Sorensen et al. (38) carried out a parallel-design intervention during which 40 overweight men and women consumed daily supplements containing sucrose (between 650 to 900 kcal/d depending on body weight) or artificial sweeteners, primarily in the form of beverages. After 10 weeks, body weight had increased and CRP had increased modestly but non-statistically significantly (6% increase) in the sucrose group compared to the artificial sweeteners group (26% decrease) (38). Adjustment for changes in body weight and energy intake did not substantially change the result, however sucrose intake appeared to have only a minor, if any, effect on CRP. In contrast, Aeberli et al. (39) conducted a randomized, crossover design intervention and measured CRP and LDL particle subclasses in 29 normal weight volunteers who consumed 40 and 80 g/d of fructose, glucose, or sucrose-sweetened beverages for 3 weeks, in addition to their habitual diet. Subjects also underwent a control, 'low fructose' phase during which fructose-containing foods and beverages were avoided. Fasting CRP concentrations were statistically significantly elevated above baseline values after all interventions and there was no statistically significant difference in CRP among the beverage groups. Additionally, a more atherogenic LDL subclass distribution was observed when subjects consumed the sucrose and fructose beverages compared to the other intervention phases (39). Interestingly, body weight increased statistically significantly during the moderate-glucose phase only, again suggesting that SSBs contribute to increased CVD risk independent of an increase in adiposity. In a longer term RCT, Maersk et al. (40) investigated the effect of different beverages on fat accumulation in the liver, muscle, and visceral depot, as well as the influence on blood lipid concentrations. Forty-seven subjects were randomized in a parallel design to consume 1 L/d of either regular soda, skim milk, artificially-sweetened soda, or water for 6 months. At the end of the intervention, the change in total fat mass and body weight was not different among groups, however the relative change between baseline and follow-up in

liver, skeletal muscle, and visceral fat were all statistically significantly greater in the soda group compared to the other three beverage groups. Fasting triglycerides and total cholesterol were also highest in the soda group (40), lending further support to the hypothesis that SSBs contribute to CVD risk by mechanisms partially independent of an increase in total body weight and fat mass.

Given the evidence from observational and intervention studies, as well as a continually evolving understanding of the processes occurring within adipose tissue that promote dysfunction, there is good reason to implicate SSBs as a central player in the pathogenesis of cardiometabolic disease, both through increased adiposity and also body weight-independent mechanisms, possibly including low-grade inflammation.

1.3. Mechanisms linking sugar-sweetened beverages to obesity and cardiometabolic disease

It is clear that the relationship between SSB intake and chronic disease is at least partly mediated by adiposity. There are several potential mechanisms to consider that could explain the link between SSB intake and obesity, as well as SSB intake and cardiometabolic disease in the absence of obesity, such as the liquid form in which SSBs are consumed or the metabolic impact of the component sugars individually (Figure 1.1). Disentangling these processes requires studies designed specifically for this purpose. As just one study cannot definitively answer all the open questions, each study must be considered within the framework of the larger body of literature on the topic. First, the mechanism of inflammation will be examined as a link between SSB intake, adiposity, and cardiometabolic disease risk, followed by a discussion of the mechanisms that promote a positive energy balance leading to weight gain in the context of SSB consumption. Lastly, an evaluation of the mechanisms that might promote an increase in cardiometabolic disease risk in the absence of weight gain will be considered in an effort to identify what is known and what open questions remain in the literature regarding the influence of SSBs on energy homeostasis, inflammation, and chronic disease risk.

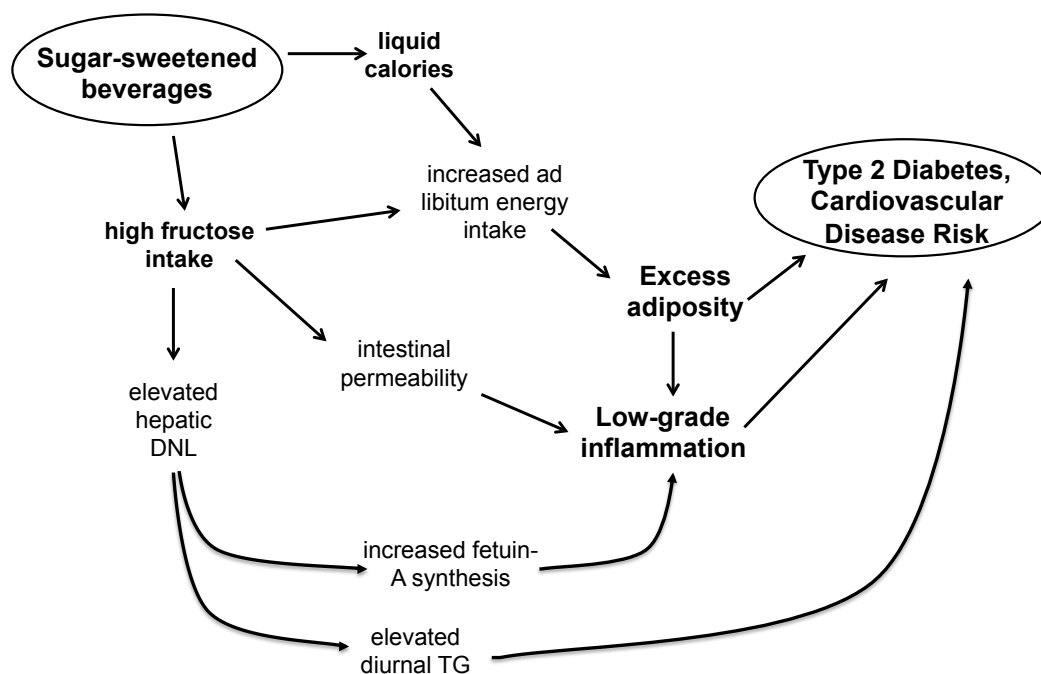


Figure 1.1. Conceptual paradigm outlining the hypotheses motivating this study.

Sugar-sweetened beverage intake is associated with chronic disease through adiposity dependent mechanisms, such as increased energy intake, and also potentially through adiposity independent mechanisms, such as low-grade chronic inflammation. This study was designed to determine whether a specific effect of fructose is responsible for the association between SSB consumption and biomarkers of chronic disease through mechanisms, such as increased hepatic de novo lipogenesis or increased intestinal permeability, that lead to an increase in low-grade systemic and adipose tissue inflammation; or if the associations are mediated by an increase in body weight due to excess energy intake triggered by a blunted satiety response to fructose or to the liquid form in which SSBs are consumed.

1.3.1. Mechanism 1: Low-grade, chronic inflammation as potential major link between sugar-sweetened beverage intake, adiposity, and cardiometabolic disease risk

Initially elucidated from rodent models of obesity, there is now widespread evidence from human studies to suggest that low-grade inflammation is associated with obesity and is causally linked to cardiometabolic disease (36). Specifically within adipose tissue, it appears that inflammation is initially triggered as an adaptive response to chronic, excessive energy intake, which promotes adipose tissue expansion to accommodate the storage of triglyceride. Tissue expansion stimulates the infiltration of macrophages and other leukocytes, which in turn secrete

pro-inflammatory cytokines which perpetuate the inflammatory process (41). Tissue expansion also stimulates angiogenesis because the existing blood supply becomes insufficient to support the tissue, and mild hypoxia results (42). If new blood vessel formation is unable to resolve the hypoxia, an inflammatory response is activated in this manner as well (43). Transcription factors such as nuclear factor kappa β (NF- $\kappa\beta$) and hypoxia-inducible factors (HIF-1a) promote the expression of proinflammatory cytokines, such as tumor necrosis factor (TNF) α and interleukin (IL)-6, in adipocytes and macrophages residing in the adipose tissue (43). Indeed, obesity is associated with increased expression of inflammatory genes in adipose tissue (44), as well as higher concentrations of activated immune cells in the stromavascular (SVC) fraction isolated from the adipose tissue of obese compared to lean humans (45).

Many factors influence the progression from obesity to insulin resistance and cardiometabolic disease and there is considerable interindividual variation in the adiposity mass at which metabolic dysfunction occurs. However, a wide body of epidemiological, clinical, and experimental evidence supports the statement that inflammation is causally linked to cardiometabolic disease (36). One hypothesis suggests that the inflammatory process is initiated by expanding adipose tissue, and a positive feedback cycle is created as cytokines recruit pro-inflammatory leukocytes to the tissue which, in turn, secrete additional cytokines and dampen insulin signaling. Specifically, TNF α interferes with tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1), which promotes adipose tissue-specific insulin resistance (36, 46-48). This is further exacerbated by the downregulation of the insulin-sensitizing adipokine adiponectin and the increased expression of resistin. Resistin has been implicated as a key player in obesity-induced insulin resistance and is also expressed by peripheral blood mononuclear cells (PBMCs) and adipose tissue macrophages (ATMs). It is potently induced in response to TNF α and IL-6 and serves to further perpetuate the pro-inflammatory milieu (49). Combined, a chronic state of inflammation is also sustained by the progressively worsening ability of adipose tissue to respond to insulin. This results in both the blunted ability of adipocytes to take up fatty acids, as well as the incomplete inhibition of lipolysis by insulin in the postprandial state leading to deposition of fatty acids into other non fat-storage organs, such as liver and muscle, which is a hallmark of metabolic disease (50). This process has been observed in mouse models of obesity (36, 45) and data from human studies also

strongly support the role of adipose tissue inflammation in the pathogenesis of insulin resistance (51-55). Adipose tissue inflammation is also strongly associated with systemic inflammation (56), which offers another pathway by which adipose tissue influences the etiology of chronic disease.

Chronic, low-grade systemic inflammation is characterized by CRP, IL-6, and other pro-inflammatory cytokines circulating at concentrations above 'healthy normal', but below ranges that would be diagnostic of an acute illness or bacterial infection (57). Obesity is now considered a chronic inflammatory state (58) and the inflammatory process plays a central role in the etiology of many of the comorbidities associated with obesity, such as CVD (36). Indeed, obese individuals have more than a 2-fold higher risk of developing CVDs compared to lean individuals (59). The events surrounding the development of atherosclerosis, such as the stimulation of coagulation, fibrinolysis, hypertension, and oxidative stress, are all mediated through pro-inflammatory-cytokine signaling (60). Circulating resistin is correlated with plasma CRP, TNF α , and IL-6, and is secreted from macrophages. Resistin specifically promotes endothelial dysfunction, vascular smooth muscle cell proliferation, arterial inflammation, and the formation of foam cells which are key events in the pathogenesis of atherosclerosis (61). C-reactive protein is a validated biomarker of CVD risk independently of adiposity and other traditional risk factors, such as dyslipidemia, because of the strong association between elevated CRP and the prediction of future coronary heart disease (62). Yet, the underlying causes contributing to this persistent, low-grade inflammation are not entirely understood. It could be that lifestyle factors, such as poor diet or lack of exercise, serve to maintain a chronic inflammatory state. It follows then, that these and other factors that promote excess energy intake and obesity are also likely associated with CVD and metabolic disease risk and mediated through inflammation.

1.3.2. Mechanism 2: Increased energy intake and adiposity due to inadequate compensation for liquid calorie intake

The consensus opinion on how SSB consumption might increase body weight is that calories consumed in liquid form are treated differently in the body than foods consumed in solid form (63). Indeed, large observational studies suggest that children who obtain a greater proportion of their total energy from liquid calories are more likely to be overweight compared to children who

consume the majority of their energy from solid food calories, suggesting that liquid energy intake is a predictor of childhood obesity (64).

Early intervention studies in this area have shown that different types of caloric beverages, such as beer and soda, trigger an overall increase in energy intake within a single meal (65). In one crossover intervention, women given a solid food lunch to be consumed *ad libitum* in addition to either water, diet soda, regular soda, orange juice, 1% milk, or no beverage consumed an additional 104 kcals, on average, when also consuming a calorie-containing beverage (156 kcal) (66). This suggests that subjects were able to compensate for only $\sim 1/3$ of the calories obtained in liquid form. Notably, this occurred in the absence of any difference in self-reported satiety ratings suggesting that calories consumed in liquid form add greater energy to a meal without influencing satiety compared to isocaloric solid foods.

Similarly, a crossover design intervention in lean and obese men sought to compare the satiety effect of calorically-matched foods of identical macronutrient composition when they are delivered in solid versus liquid form (67). For instance, the effect of consuming watermelon slices versus watermelon juice or solid coconut meat versus liquid coconut milk on appetite and energy intake was determined. Regardless of macronutrient composition, subjects consistently consumed statistically significantly greater overall energy on days when the liquid form of the food was consumed compared to the solid form (67). This suggests that foods consumed in liquid form elicit weaker satiety signals than solid foods even when the foods are identical in energy and composition. This effect was confirmed in a separate study during which subjects consumed identical isocaloric foods delivered in solid, semi-solid, and liquid form and the interval until the next meal was initiated was measured. The interval was shortest following consumption of the beverage, followed by the semi-solid, and longest following the solid condition (68) which again suggests that there is greater risk of excess energy intake when beverages, rather than solid foods, are consumed. Put another way, perceived satiety appears to be blunted following the consumption of liquid versus solid calories even when food items are equivalent in calories and macronutrient composition.

In regard to SSBs specifically, the first human study comparing the effects of consuming HFCS-sweetened- vs. aspartame-sweetened beverages on total energy intake and body weight was carried out by Tordoff and Alleva in 1990 (69). Subjects were asked to consume 1135 g of either

aspartame-sweetened or HFCS-sweetened (equivalent to 530 kcal) beverages each day for 3 weeks in addition to their habitual diet. During the intervention, subjects increased their total energy intake by 13% on average above their baseline intake when consuming the HFCS-sweetened beverages, compared to the aspartame group, which resulted in a statistically significant increase in body weight. Interestingly, subjects in the aspartame group decreased their total energy intake by 7% of their baseline intake during the intervention period, which indicates that all subjects decreased their energy intake from solid foods to a similar degree regardless of whether the beverage contained calories (69). This finding supports the earlier conclusion that consuming sugars in liquid form blunts satiety mechanisms which leads to incomplete compensation for liquid calories and results in excess total energy intake.

Similarly, Reid et al. (70) randomized normal-weight women to receive either aspartame or 430 kcal of a sucrose-flavored beverage each day for 4 weeks, in addition to their habitual diet. Overall, the sucrose group consumed on average 190 kcal greater total energy each day compared to their baseline intake, while the aspartame group decreased their overall energy intake slightly compared to their baseline intake. This effect resulted in a small increase in body weight in the sucrose-beverage group, but the result was not statistically significant likely due to large intra-individual differences in weight fluctuation over the course of this relatively short study (70).

Lastly, Raben et al. (71) asked overweight subjects to consume either 600 kcal/d of sucrose-containing solid foods and beverages or the equivalent amount of artificially-sweetened products in addition to their habitual diet for 10 weeks. During the parallel intervention period, total energy intake increased by approximately 360 kcals/d above baseline in the sucrose group while the artificial sweeteners group maintained their baseline intake. As a result, subjects in the sucrose group gained a statistically significant amount of weight (1.6 kg) and fat mass, while subjects in the artificial sweeteners group lost on average 1.0 kg over the course of 10 weeks. Because this study provided sucrose products in both beverage and semi-solid form, the true compensatory effect of liquid sugar calories on overall energy intake could not be calculated. However, 70% of the products were provided as soda, which suggests that the observed overconsumption of energy in the sucrose group may be attributable to a failure of liquid calories to elicit the same energy intake regulatory mechanisms as solid food calories. Indeed, there were

no statistically significant differences between the groups in terms of self-reported hunger, fullness, or palatability of the foods despite the statistically significant difference in energy intake (71).

Perhaps the strongest evidence of a differential effect of sugars consumed in liquid vs. solid form on overall energy intake and body weight was provided by DiMeglio and Mattes (72). In their randomized, controlled, crossover intervention, healthy subjects consumed 450 kcal/d in the form of either jellybeans or soda in addition to their habitual diet for 4 weeks each. During the solid sugar phase (jellybeans), subjects completely compensated for the calories consumed as jellybeans by reducing their intake of solid foods by an equivalent amount. However, during the soda phase, subjects failed to decrease their intake of solid foods, which remained at an amount equivalent to their habitual intake and resulted in statistically significant body weight gain during the liquid soda phase only (72). The strength of this study lies in the crossover design and also in the well-matched composition of the solid vs. liquid sugar products (each containing roughly 50% glucose and 50% fructose) suggesting that the observed increase in energy intake and body weight can be explained by the liquid nature of the sugar.

In an attempt to offer a mechanistic explanation for the observed effect of liquid calories on body weight, Cassady et al. (73) enrolled 52 healthy adults into a randomized, crossover intervention comparing the sensory and cognitive responses of consuming calories in liquid vs. solid form. Appetite, gastric-emptying, orocecal transit times and endocrine responses were all monitored for 4 hours following consumption of an isocaloric and macronutrient-matched liquid vs. solid meal. Following the liquid meal, subjects reported greater hunger and lower fullness sensations and the rate of gastric emptying and orocecal transit were statistically significantly greater compared to the solid-meals group. Following the liquid meals, insulin and glucagon-like peptide (GLP)-1 release were attenuated and ghrelin suppression was blunted, also to a statistically significant degree, compared to the response following intake of a solid meal. This resulted in greater overall energy intake on days when liquid meals were consumed compared to solid meals (73).

Taken together, cognitive processes, gastric emptying, intestinal transit, nutrient absorption rate, endocrine responses, and other sensory factors are all modestly to substantially altered when

liquid calories are consumed compared to solid foods (73-75). Over time, chronic consumption of liquid calories would likely result in excess energy intake and weight gain.

1.3.3. Mechanism 3: Increased energy intake due to inadequate satiety response to fructose

On the other hand, the observed relationship between SSB consumption and increased energy intake and weight gain might also be explained by the fructose component of the SSB. Sugars used in SSBs are a combination of fructose and glucose occurring in a ratio of 60:40 fructose: glucose in the most commonly consumed sweetened beverages (9). Consuming fructose, perhaps in any form, might be less effective at inducing an appropriate satiety response compared to glucose. This hypothesis is based largely on the fact that fructose does not trigger an insulin response equivalent to that of glucose (76). Insulin is an important adiposity hormone involved in the long-term regulation of energy homeostasis. In the postprandial period, insulin-mediated uptake of glucose into adipocytes triggers an increase in the production of the other key adiposity hormone, leptin (77). Insulin also suppresses the production of the major orexigenic hormone, ghrelin, from the stomach (76). Ghrelin is an acute trigger of food intake, controlling short-term energy intake, while insulin and leptin play a key role in the long-term regulation of energy homeostasis (78). In response to a chronic excess in energy intake, the adiposity signals insulin and leptin increase as body fat mass expands, and work to counteract energy intake by decreasing hunger cues. Leptin is particularly important as it allows for an enhanced response to satiety signals released from the GI tract, such as cholecystikinin (CCK), peptide (P)YY and GLP-1 in the hindbrain, while impaired leptin signaling causes reduced sensitivity to these signals thereby increasing meal size (79). The interaction between leptin signals from the hypothalamus and satiety signals are integrated in the hindbrain to influence food intake and energy expenditure and allows for the short-term adjustment of individual meals based on changes in body fat mass (80). Normally, the ingestion of glucose leads to a postprandial rise in peripheral blood glucose and insulin levels, a small increase in postprandial leptin levels, and suppression of ghrelin concentrations in plasma. In contrast, fructose does not trigger a corresponding increase in postprandial insulin levels, and therefore also fails to increase leptin or suppress ghrelin concentrations (76). This important finding was determined from an in-patient study investigating the hormonal response to consuming glucose- vs. fructose-sweetened beverages in the context of a normal diet. Twelve normal-weight women consumed 30% of their calories in the form of an SSB in addition to solid foods in a randomized, crossover design

intervention. Over the course of 24-hours, blood samples were collected every 30-60 minutes and diurnal glucose, insulin, leptin, and ghrelin values were calculated. Notably, statistically significant differences in insulin, leptin, and ghrelin responses following glucose vs. fructose consumption were observed even within 24-hours. Ghrelin was suppressed by 30% within 2 hours of glucose consumption and was statistically significantly blunted following fructose consumption, and the area under the 24-hr curve for leptin was reduced by 21% during the fructose phase compared to the glucose phase (76). This result was somewhat surprising given that insulin and leptin were previously thought to function primarily in the long-term regulation of energy balance. These data support the hypothesis that fructose may be less satiating than glucose, resulting in acute changes in meal frequency, and/or meal size, and thus could lead to excess calorie intake and increased adiposity over time.

1.3.4. Mechanism 4: Adiposity-independent effects of fructose on risk factors for cardiometabolic disease

Additional experimental evidence also suggests that fructose uniquely influences cardiometabolic disease risk factors compared to glucose. While several studies have investigated the effect of fructose- vs. glucose-sweetened beverages on energy homeostasis and energy intake (39, 81, 82), these studies suffered from important methodological limitations, as further discussed below. Additionally, the exact mechanism explaining the observed association between SSB intake and T2DM and CVD independently of the increased risk conferred by excess body fat remains elusive (7, 32, 83). Specifically, it has remained largely unclear whether excessive fructose consumption from SSB has acute, i.e. weight gain independent, effects on cardiometabolic risk factors.

Several studies have shed light on specific effects of fructose as compared to glucose. In a parallel design intervention, Stanhope et al. (81) provided beverages sweetened with either glucose or fructose to matched overweight and obese individuals for 10 weeks. Subjects were asked to consume 4 servings of an SSB daily, which provided 25% of their estimated energy requirements in addition to their habitual diet, which was consumed *ad libitum*. Weight gain on both diet arms was similar suggesting no difference between fructose- and glucose-sweetened beverages on energy homeostasis. However, despite similar weight gain, only the fructose group experienced an increase in visceral adipose tissue, hepatic *de novo* lipogenesis (DNL), diurnal triglyceride concentrations, and circulating small dense LDL particles (81). In addition, net

postprandial fat oxidation was decreased following the fructose compared to the glucose-beverage period and resting energy expenditure decreased statistically significantly from baseline (84). These physiological changes among the subjects consuming fructose-sweetened beverages also corresponded to an increase in fasting glucose and insulin and a decrease in overall insulin sensitivity. This indicates that in addition to promoting caloric excess and weight gain, fructose in particular had effects on cardiometabolic risk factors independent of increased adiposity. What remains unclear, however, is whether the fructose effects were dependent upon overfeeding and weight gain.

Fructose promotes intrahepatic lipid accumulation: Some of the aforementioned effects likely result from the fact that fructose is rapidly removed from the blood via the portal vein and, unlike glucose, is metabolized by the liver where it stimulates DNL (85). This may promote intra-abdominal and ectopic fat accumulation, insulin resistance, and dyslipidemia to a greater extent than does glucose. This may be a key point linking fructose in particular to the pathogenesis of T2DM in a body weight-independent manner as accumulation of fat in the liver appears to be a better predictor of the development of insulin resistance than visceral adipose tissue volume (86). Much of the previous work in this area has concluded that the deleterious effects of fructose occur only in the presence of overfeeding (87) and weight gain. Therefore, studies examining the effects of a high-fructose diet on liver fat, DNL, and CVD risk factors possibly related to liver fat, such as hepatic insulin resistance and diurnal hypertriglyceridemia, in the absence of overfeeding are necessary.

Several studies have begun to shed light on this important distinction. For example, Hochuli et al. (88) asked 34 free-living, healthy men to consume beverages sweetened with fructose, glucose, or sucrose in addition to their habitual diet for 3 weeks. In a crossover design, each subject consumed beverages sweetened with 80g (high) or 40g (medium) fructose, or 80g/d sucrose or glucose in random order. Mean total energy intake did not differ during any of the intervention periods compared to baseline. However, plasma palmitate concentrations, a marker of DNL, were statistically significantly higher following both the low and moderate fructose phases compared to the glucose and sucrose phases (88). In a subset of 9 subjects from that study, Aeberli et al. (89) conducted a hyperinsulinemic-euglycemic clamp after each beverage phase and discovered that suppression of hepatic glucose production was statistically

significantly lower following the high fructose phase compared to the glucose and sucrose phases (89). This reinforces the concept that the development of hepatic insulin resistance, a risk factor for cardiometabolic disease, is uniquely triggered by moderate amounts of fructose compared to glucose in a weight-gain independent manner.

Schwarz et al. (90) carried out a calorie-controlled, inpatient intervention which also nicely illustrated this point. The investigators carried out a crossover design, 9-d inpatient intervention study designed to maintain subjects' body weight while also providing 25% of their energy needs in the form of either a fructose-sweetened beverage or an isocaloric amount of complex carbohydrates (90). At the end of the intervention, all subjects had exhibited statistically significantly greater rates of DNL and liver fat content during the fructose-phase compared to the complex carbohydrate phase. Furthermore, during the hyperinsulinemic clamp, hepatic glucose production was not suppressed to the same degree following the high-fructose diet suggesting that the high-fructose feeding had induced hepatic insulin resistance (90). This was the first controlled study that was not confounded by weight gain (i.e. isocaloric conditions), to suggest that fructose differentially affects liver fat, DNL, and hepatic insulin sensitivity compared to glucose-based carbohydrates under conditions of weight stability.

Hypothesis: fructose-induced hepatic lipid accumulation promotes adipose tissue inflammation via fetuin-A: As previously mentioned, adipose tissue inflammation features prominently in the pathogenesis of metabolic disease. One novel mechanism linking fructose-induced hepatic DNL to AT inflammation could occur through the production of the hepatokine fetuin-A. Fetuin-A is a liver-derived glycoprotein that was initially discovered as an endogenous inhibitor of the insulin receptor in skeletal muscle and liver (91). More recently, it was observed that circulating concentrations of fetuin-A are elevated in humans with excess liver fat (92) and are also strongly correlated with markers of atherosclerosis (93) and T2DM (94), all independently of body adiposity. It has also been known that saturated fatty acids (SFAs) can induce inflammation in adipose tissue via toll-like receptor (TLR)4 signaling but because SFAs do not bind directly to TLR4, the mechanism for activation was unknown (95). Recently, however, studies in rodents have shown that fetuin-A acts as a chaperone allowing SFAs to bind to TLR4 and activate inflammatory pathways in adipocytes and macrophages (96). This has since been confirmed in

humans as well; the SFA palmitate binds with high affinity to fetuin-A and induces a strong proinflammatory response in adipocytes (95).

Further supporting this concept, elevated circulating concentrations of both fetuin-A and FFA are strongly associated with and predictive of insulin resistance (97), which is intriguing given that a major etiologic factor in the development of insulin resistance is AT inflammation (55). It seems plausible that fetuin-A concentrations may also increase in response to consuming fructose-sweetened beverages given that excessive fructose induces hepatic DNL. The combination of elevated fetuin-A and FFAs could trigger low-grade AT inflammation, offering a mechanism by which fructose might directly promote AT inflammation.

Hypothesis: fructose promotes systemic inflammation via increased intestinal permeability: Not only can AT inflammation promote systemic inflammation (56), but there is strong evidence from animal models to suggest that fructose might contribute directly to low-grade systemic inflammation through other mechanisms. Specifically, it has been proposed that fructose might trigger systemic inflammation by promoting endotoxemia through an increase in intestinal permeability. In rodent models, excessive fructose consumption led to hepatic and systemic inflammation via an increase in intestinal permeability in the proximal small intestinal tract (98, 99). In these experiments, fructose- but not glucose- consumption led to a disassembly of the tight junctions and increased translocation of bacterial lipopolysaccharide (LPS) into the portal vein. Elevated portal vein LPS triggered inflammation in the liver via a mechanism that was dependent on the LPS-receptor (TLR4) as well as the presence of bacteria in the gut (98).

A similar study carried out in primates showed that monkeys fed an *ad libitum*, high fructose diet (24% of energy) compared to monkeys fed a low-fructose, low-fat diet for several years (100) developed diabetes and hepatic steatosis more frequently than those on normal chow. In those animals consuming a high fructose diet that was calorically controlled to prevent weight change, histologic markers of liver damage increased within 6 weeks. Inflammation, as measured by CRP and plasma endotoxin was also statistically significantly increased as was plasma soluble CD14 receptor and LBP, both markers of bacterial presence (100). The combined evidence suggests that fructose feeding, even under short-term, calorically controlled conditions, promotes systemic inflammation through the translocation of bacteria or bacterial antigens from the gut lumen to the portal circulation.

Direct evidence of the impact of fructose on systemic inflammation in humans is lacking. Only a few studies have measured markers of systemic inflammation in response to fructose consumption and the results were not conclusive. In a randomized, crossover design study investigating the effect of acute fructose vs. glucose vs. sucrose feeding, Jameel et al. (101) provided 14 healthy subjects with 50 g (200 kcal) of sugar in beverage form after an overnight fast and measured CRP concentrations every 30 min for 2 hours. At 30 min, CRP was statistically significantly greater following fructose intake compared to both the glucose and sucrose beverages. The overall 2-hr area-under-the curve (AUC) for CRP was statistically significantly greater following the fructose vs. glucose phase, but not the fructose vs. sucrose phase (101). This suggests that in the short-term, fructose might promote an immediate but transient inflammatory response, however the longer-term clinical relevance of this finding is unclear.

Aeberli et al. (39) have been the only group to report a statistically significant effect of glucose and fructose-sweetened beverages on plasma CRP. Twenty-nine, normal weight volunteers underwent a randomized, crossover intervention comparing the effects of 40 and 80 g/d of fructose, glucose, or sucrose-sweetened beverages on plasma CRP. Subjects consumed the beverages for 3 weeks in addition to their habitual diet, and also underwent a 'low fructose' phase during which fructose-containing foods and beverages were avoided. Body weight increased statistically significantly during the moderate-glucose phase only, but percent body fat increased statistically significantly during the high-fructose intervention only. Following each diet phase, including the low-fructose phase, CRP concentrations were statistically significantly elevated above baseline values, and there was no statistically significant difference in CRP among the intervention groups (39). This was the first controlled intervention to measure a statistically significant effect of SSB consumption on CRP; however, because levels were also elevated above baseline following the low-fructose diet phase, this study does not provide clear evidence that SSB or fructose trigger systemic inflammation.

In a parallel-design study, Silbernagel et al. (102) provided 20 healthy subjects with 150 g/d (600 kcal) of either fructose or glucose in liquid form to be consumed along with their habitual diet. After 4 weeks, body weight had increased statistically significantly from baseline in the glucose group only, but visceral and liver fat were not statistically significantly different from baseline in

either group. Furthermore, circulating CRP, PAI-1, MCP-1, and E-selectin, also proinflammatory mediators, did not change from baseline in response to either treatment (102).

Similarly, Cox et al. (103) observed no changes in plasma CRP or IL-6 in older, overweight or obese adults consuming fructose vs. glucose beverages at 25% of their estimated energy requirements for 10 weeks. However, MCP-1, PAI-1, and E-selectin all increased to a statistically significant degree from baseline following fructose- but not glucose- beverage consumption. In this study, total body weight and fat mass increased statistically significantly but similarly in both groups, but visceral adipose tissue volume increased in the fructose-beverage group only (103). As visceral, rather than subcutaneous, adipose tissue is the primary determinant of circulating MCP-1 and PAI-1 (104, 105) it is possible that the expansion of visceral adipose tissue was the primary driver of the increase in these specific markers of inflammation. This also offers an explanation for the discrepant findings between the studies by Silbernagel and Cox. Fructose- but not glucose- triggered an increase in VAT over 10-weeks of daily beverage consumption (81) but this was not the case after only 4 weeks of high fructose intake (102). Therefore, it appears that the expansion of VAT might be required for the initiation of an inflammatory response, which indicates a unique role for fructose in the promotion of systemic inflammation.

The open question remains whether fructose- compared to glucose- uniquely triggers an increase in systemic inflammation independent of a change in body weight. A discussion of the limitations of prior studies attempting to address this issue is presented below.

1.4. Gaps in knowledge

Each of the studies discussed thus far have shed light on one particular metabolic impact of SSB consumption. However, each have been limited in their ability to definitely answer the question of whether it is 1) the liquid sugar nature of the SSBs, 2) the fructose component of the SSB, or 3) a combination of both: fructose consumed in liquid form, that is ultimately responsible for the deleterious health outcomes associated with SSB consumption. For example, studies designed to test the liquid calories hypothesis have assessed *ad libitum* energy intake while subjects also consumed calorically matched solid vs. liquid forms of sugar (jellybeans vs. soda) (72). Because subjects consumed greater overall energy and gained weight while consuming the soda compared to the jellybeans, this study provides strong evidence for the liquid calories hypothesis. However,

because the test foods both also contained fructose, this study cannot determine (a) whether energy consumption was elevated in both intervention arms compared to a low-fructose condition; and (b) whether it was the liquid sugars, or specifically the fructose consumed in liquid form, that was driving the increase in energy intake and body weight. In order to answer these questions, the study design would have needed to include two additional arms: one arm providing solid low-fructose foods, and one arm providing liquid low-fructose beverages, in addition to the liquid and solid high-fructose arms.

On the other hand, studies designed to test the fructose hypothesis have isolated fructose from glucose, both in liquid form, and assessed differences in the impact of consuming these two sugars in the context of an ad libitum diet on energy homeostasis as well as glucose and lipid metabolism (81). The outcomes showed that those who consumed fructose beverages displayed early signs of dyslipidemia, hepatic DNL, and decreased insulin sensitivity compared to those who consumed glucose beverages. However, in this case, the study was not a crossover design, the participants were all overweight or obese prior to enrollment, the participants gained weight over the course of the intervention, and a fructose malabsorption test was not included in the screening procedures, which will be discussed below. Therefore, the question of whether fructose-sweetened beverages differ from glucose-sweetened beverages in their impact on energy homeostasis, and the weight gain-independent metabolic effects of fructose consumption cannot be determined from this type of study.

Lastly, a study designed to determine the effect of fructose on hepatic DNL and lipids in a weight gain-independent manner utilized a crossover design, successfully maintained subjects' body weight, and provided all foods for the duration of the study period (90). However, in this case, a fructose beverage was compared to an isocaloric amount of non-fructose carbohydrate consumed in solid form. Therefore, it cannot be determined whether it was the liquid form in which the sugar was consumed, or the fructose itself, or the combination of both, that was responsible for the negative outcomes observed during the fructose phase. This study also did not include procedures to screen for fructose malabsorption prior to subject enrollment.

In fact, no studies discussed thus far have included fructose malabsorption tests. Fructose, when consumed in a molar ratio of greater than 1 relative to glucose, is malabsorbed in as much as 50% of the population (106). This is because fructose is absorbed by carrier-mediated facilitated

diffusion, and the absorptive capacity for fructose cleaved from sucrose exceeds that of pure fructose in healthy individuals (107). Glucose enhances fructose uptake in a dose dependent manner, which explains why healthy individuals do not display symptoms of fructose malabsorption when consuming high amounts of sucrose or HFCS. Fructose malabsorption is normally diagnosed by hydrogen breath test following consumption of a fructose load. The presence of non-absorbed carbohydrates results in bacterial fermentation in the small intestine. Fermentation leads to the production of short-chain fatty acids, hydrogen, carbon dioxide, and trace gases. As hydrogen is not metabolized by humans and must be excreted in the breath, this offers a means by which fructose absorption can be assessed. In general, a rise in breath hydrogen of greater than 20 ppm above baseline values reflects incomplete absorption (106). While pure fructose is not widely consumed in the average diet in absence of glucose, fructose malabsorption could be an important confounder in randomized trials aimed at determining the physiological differences between glucose and fructose consumption. Because none of the studies discussed thus far screened for fructose malabsorption, it is likely that a substantial number of participants were in fact fructose malabsorbers. As a result, these studies may have underestimated the true effect of fructose on energy intake, energy homeostasis, and body weight, and may also explain the discrepant findings in studies assessing the impact of fructose on other endpoints including systemic inflammation.

The overall goal of work in this area is to inform public health recommendations surrounding the intake of sugar sweetened beverages and dietary fructose in particular. Given the lack of consensus from randomized trials as well as recommendations across different government agencies, it is clear that outstanding questions concerning the impact of added sugars on health still exist. For example, the Institute of Medicine recommends no more than 25% of total calories should come from added sugar (108), the American Heart Association Nutrition Committee suggests that women consume no more than 100 kcal/d and men no more than 150 kcal/d of added sugar (109) and the updated Dietary Guidelines for Americans 2015 decreased the recommendation to no more than 10% of total energy, down from 25% recommended in 2010 (8). Even if consensus is increasing to limit total sugar intake, it has remained unclear whether limiting fructose-rich sugars should be a major focus, i.e. whether switching to sweeteners containing higher proportions of glucose would offer health benefits over the current widely used sugars that provide fructose and glucose in roughly equal proportions. In light of this, and in an

attempt to address the limitations of prior studies in this area, we aimed to disentangle the effects of fructose from those of glucose when consumed in liquid form in a weight gain-independent manner, and in the context of an ad libitum diet in individuals free from fructose malabsorption.

1.5. Objective

We carried out a randomized, controlled, dietary intervention trial, with a double-blind cross-over design, to test the effects of beverages sweetened with fructose, glucose, or high fructose corn syrup (HFCS, 55% fructose and 45% glucose) on low-grade chronic inflammation and energy intake. Twenty-four men and women (12 normal weight, 12 overweight or obese) completed three independent 8-day dietary periods. During each of the dietary phases, subjects consumed four servings per day of a SSB containing either fructose, glucose, or HFCS in addition to standardized solid food consumed *ad libitum*. This study addressed the following

Primary Specific Aim:

- (1) To compare the effects of fructose- vs. HFCS- vs. glucose-sweetened beverages on biomarkers of systemic inflammation, as assessed by measuring CRP and IL-6 concentrations in fasting plasma before and after each diet phase.** We hypothesize that plasma CRP and IL-6 concentrations will be higher after participants have consumed the fructose-sweetened beverages as compared to the glucose-sweetened beverages. We further hypothesize that plasma CRP and IL-6 concentrations will be intermediate when participants consume the HFCS-sweetened beverage.

We also addressed the following **secondary aims:**

- (2) To assess whether the intake of fructose- vs. HFCS- vs. glucose-sweetened beverages promotes adipose tissue inflammation, as assessed by the adipose tissue expression of adiponectin and TNF α .** We hypothesize that the gene expression level of TNF α will be increased and that of adiponectin will be reduced in the adipose tissue following consumption of diets containing fructose-sweetened beverages as compared to diets containing glucose-sweetened beverages. We further hypothesize that adipose tissue TNF α and adiponectin gene expression levels will be intermediate when participants consume the HFCS-sweetened beverage.

- (3) To assess whether the consumption of fructose- vs. HFCS- vs. glucose-sweetened beverages promotes intestinal permeability as measured by the urinary lactulose-mannitol test and plasma zonulin and lipopolysaccharide-binding protein (LBP) concentrations.** We hypothesize that the ratio of lactulose to mannitol in urine and the concentration of zonulin and LBP in plasma will be higher after participants have consumed the fructose-sweetened beverages as compared to the glucose-sweetened beverages. We further hypothesize the ratio of lactulose to mannitol in urine and the concentration of zonulin and LBP in plasma will be intermediate when participants consume the HFCS-sweetened beverage.
- (4) To compare the effects of fructose- vs. HFCS- vs. glucose-sweetened beverages on the hepatokine Fetuin-A.** We hypothesize that the hepatokine Fetuin-A will be higher after participants have consumed the fructose-sweetened beverages as compared to the glucose-sweetened beverages. We further hypothesize that Fetuin-A will be intermediate when participants consume the HFCS-sweetened beverage.
- (5) To compare the effects of fructose- vs. HFCS- vs. glucose-sweetened beverages on *ad-libitum* energy intake.** We hypothesize that total energy intake will be higher when participants will consume the fructose-sweetened beverages as compared to the glucose-sweetened beverages. We further hypothesize that total energy intake will be intermediate when participants will consume the HFCS-sweetened beverage.

1.6. Public Health Implications of Research

These studies incorporate rigorous measures to determine whether glucose and fructose, consumed in the context of SSBs, differentially impact health. If fructose leads to greater overconsumption of calories and/or low-grade chronic inflammation than glucose, the public health implications could be highly relevant. Because fructose intake is a modifiable dietary factor, strategies to reduce consumption could provide one promising approach to combat the epidemic of obesity and associated cardiometabolic diseases. This study also has the potential to lead to new hypotheses about the mechanisms by which diet composition may be linked to chronic inflammation.

2. NO EVIDENCE OF A DIFFERENCE IN AD LIBITUM ENERGY INTAKE IN HEALTHY MEN AND WOMEN CONSUMING BEVERAGES SWEETENED WITH FRUCTOSE, GLUCOSE, OR HIGH-FRUCTOSE CORN SYRUP: A RANDOMIZED TRIAL

Increased energy intake is consistently observed in individuals consuming sugar-sweetened beverages (SSBs), likely mainly due to an inadequate satiety response to liquid calories. However, SSBs have a high content of fructose, the consumption of which acutely fails to trigger responses in key signals involved in energy homeostasis. It is unclear whether the fructose content of SSBs contributes to the increased energy intake in individuals drinking SSBs. We investigated whether the relative amounts of fructose vs. glucose in SSBs modifies *ad libitum* energy intake over 8 days in healthy adults without fructose malabsorption. We conducted two randomized, controlled, double-blind cross-over studies to compare the effects of consuming four servings per day of a fructose-, glucose-, or aspartame-sweetened beverage (Study A, n=9) or a fructose-, glucose-, or high-fructose corn syrup (HFCS)-sweetened beverage (Study B, n=24) for eight days on overall energy intake. Sugar-sweetened beverages were provided at 25% of estimated energy requirement, or an equivalent volume of the aspartame-sweetened beverage, and consumption was mandatory. All solid foods were provided at 125% of estimated energy requirements and were consumed *ad libitum*. In Study A, *ad libitum* energy intake was $120\pm 10\%$, $117\pm 12\%$, and $102\pm 15\%$ of estimate energy requirements when subjects consumed the fructose-, glucose-, and aspartame-sweetened beverages. Energy intake was statistically significantly higher in the fructose and glucose phases compared to the aspartame phase ($p < 0.003$ for each), with no evidence of a difference between the fructose and glucose phases ($p = 0.462$). In Study B, total energy intakes during the fructose, HFCS, and glucose phases were $116\pm 14\%$, $116\pm 16\%$, and $116\pm 16\%$ of the subject's estimated total energy requirements ($p = 0.880$). In healthy adults, total 8-day *ad libitum* energy intake was increased in individuals consuming SSBs compared to aspartame-sweetened beverages. The energy overconsumption observed in individuals consuming SSBs occurred independently of the relative amounts of fructose vs. glucose in the beverages.

2.1. Introduction

Sugar-sweetened beverages provide a considerable amount of calories in the Western diet. According to current estimates, SSBs comprise 8% of daily energy intake in youth aged 2-19

years and 7% of energy intake among adults (110), the latter being equivalent to almost 40g of sugar each day. The cumulative body of evidence from randomized clinical trials and observational studies indicates that SSB consumption leads to excessive energy intake, weight gain, and an increased risk of obesity (12).

Two different mechanisms could explain why SSBs promote weight gain in healthy individuals. First, liquid calories in general (74) and SSBs in particular (63) are sensed differently by energy regulatory systems than solid foods, and humans fail to completely compensate for these calories by decreasing food intake elsewhere in the diet. Early studies on this topic have shown that including different types of caloric beverages, such as beer and soda in a meal, triggers an overall increase in energy intake within that meal (65). Less clearly established, however, is whether the type of sugar used to sweeten these beverages affects energy intake. SSBs are commonly sweetened with sucrose or HFCS, both combinations of glucose and fructose in roughly equivalent amounts. Because fructose does not stimulate insulin secretion upon ingestion, leptin and ghrelin responses are also blunted (76). This dampened hormonal signaling may result in a failure to fully engage the energy homeostasis system, thereby possibly leading to an increase in total calorie intake. This suggests that fructose may be less satiating than glucose, and perhaps a major contributing factor in the weight gain-promoting effects of SSBs considering their high fructose content. Therefore, we sought to determine whether the excess energy intake associated with SSB consumption is a result of the high fructose content of these beverages. This question may have relevance beyond SSBs, as any difference in satiety response between fructose and glucose may also extend to solid foods.

To address this question, we conducted secondary analyses on data from two controlled studies designed to determine 1) whether healthy humans can adequately compensate for calories contained in SSBs by decreasing energy intake from solid foods over a longer period of time than most previous studies, and 2) whether the degree of compensation for SSBs differs depending on the fructose content of these beverages. To our knowledge, these are the first well-controlled cross-over interventions to provide subjects with all foods for the duration of the study and to also screen for fructose malabsorption. The latter point may be critical considering that when fructose is consumed in a molar ratio to glucose of greater than 1, it is malabsorbed in as much as 50% of the population (106). Without prior screening, the metabolic and energy

homeostatic response to fructose when administered without glucose, as is the case in many artificial research settings, cannot be accurately determined.

2.2. Subjects and methods

This manuscript describes the results of two dietary interventions that were carried out consecutively at the University of Washington (UW) and the Fred Hutchinson Cancer Research Center (FHCRC) in Seattle, WA. For study A, 10 healthy, normal-weight adults [BMI (in kg/m²) between 20 and 25, age between 18 and 25 y] living in the Seattle area were included. Subjects were recruited by fliers and advertisements in the UW campus newspaper, and screened and enrolled between September 2009 and June 2011, with the final subject completing the study in August 2011. For study B, a separate group of 25 healthy adults [BMI (in kg/m²) between 20 and 40, age between 18 and 65 y] were similarly recruited by newspaper advertisements and fliers posted in the Seattle area. Subjects were recruited into two categories based on BMI: normal weight (BMI 20.0-24.9 kg/m², n=13) and overweight/obese (BMI 25.0-39.9 kg/m², n=12). Subjects were screened and enrolled between December 2011 and December 2013, with the final subject completing the study in April 2014.

In both studies, all subjects were required to have been weight stable to within 4.5 kg (10 pounds) for the previous six months and within 13.6 kg (30 pounds) of their lifetime maximum weight. Potential subjects had to be willing to consume only food and beverages provided by the research kitchen for three periods of 8 days each, and were required to be available for admission to the clinic on up to six occasions. Other exclusion criteria included smoking or the use of recreational drugs, alcohol abuse (> 2 drinks per day), a history of cardiovascular disease or diabetes mellitus, the presence or history of any chronic inflammatory, autoimmune, or metabolic disease, recent (within 12 months) pregnancy or breast feeding, the presence of phenylketonuria, hereditary fructose intolerance, or other malabsorption syndromes, presence or recent (within two months) history of anemia, and the current or recent (within 3 months) use of insulin, antidiabetics, β -blockers, glucocorticoids, anabolic steroids, warfarin, antibiotics, probiotics, or non-steroidal anti-inflammatory drugs (daily use). Before being enrolled, subjects in both studies underwent telephone and in-person screening interviews to assess medical history, obtain a plasma biochemistry panel, and conduct a fructose malabsorption test (111) to assess eligibility. Briefly, during the in-person screening visit, each subject consumed a beverage

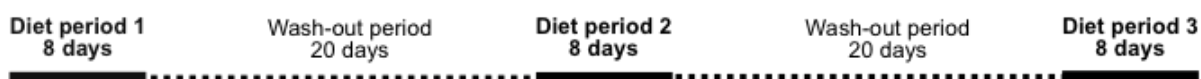
sweetened with fructose in an amount equivalent to 6.25% of estimated daily calorie intake (representing one study-beverage serving), as estimated by the Mifflin formula (112) and a standardized activity factor of 1.5. The hydrogen content of the exhaled breath was measured at baseline, 0, 30, 60, 90, 120, 150, and 180 minutes after consumption of the beverage. An increase of greater than 20 ppm above baseline for two subsequent time points was considered indicative of fructose malabsorption and the subject was excluded from the study. Written informed consent was obtained from all subjects, and studies were approved by the Institutional Review Boards (IRB) of the University of Washington (study A) and the Fred Hutchinson Cancer Research Center (study B).

2.2.1. Study design and diets

The primary aim of study A was to assess whether glucose- vs. fructose-sweetened beverages differentially influenced overall energy intake compared to aspartame-sweetened beverages. Pre-determined secondary endpoints included resting energy expenditure, fasting and post-prandial plasma concentrations of satiety and adiposity signals (leptin, ghrelin, adiponectin, insulin, glucagon-like peptide-1 (GLP-1), peptide YY, cholecystokinin, amylin, and oxyntomodulin), lipid and lipoprotein concentrations (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and free fatty acids), and plasma inflammatory mediators (C-Reactive Protein (CRP) and Interleukin (IL)-6). Of these, leptin, ghrelin, GLP-1, peptide YY, cholecystokinin, amylin, and oxyntomodulin were never analyzed due to lack of funds. No other data from this trial have been published as yet. The primary aim of study B was to assess whether glucose- vs. fructose- vs. HFCS-sweetened beverages differentially influence markers of systemic inflammation, as assessed by measuring the fasting plasma concentration of CRP and IL-6. Ad libitum energy intake was a pre-determined secondary endpoint of this study, along with fasting plasma adiponectin, zonulin, and lipopolysaccharide-binding protein (LBP), urinary lactulose/mannitol ratio, and adipose tissue inflammation. No data from this trial have been published to date. Because data from both studies together most comprehensively address the open question of whether ad libitum energy intake is differentially affected by fructose- vs. glucose-sweetened beverages, data on energy intake from both studies are presented here separately from effects of the interventions on other endpoints.

The study designs (Figure 2.1) were identical in both studies; however, the type of sweetener used during one of the diet periods was different. Study A was designed to investigate whether ad libitum calorie consumption was differentially affected by the intake of fructose-, glucose-, or aspartame-sweetened beverages. The study consisted of three separate intervention periods, each lasting eight days, and assigned in random order. During these periods, subjects consumed four servings each day of a beverage sweetened with either fructose, glucose, or a low-calorie sweetener (Equal, Merisant Company, Chicago, IL) using aspartame as the primary sweetening agent. The sweetener contains 4 kcal of energy per gram, largely from carbohydrate-based fillers used in the product; however, the amount needed to achieve a sweetness equivalent to glucose or fructose provides a much smaller amount of energy. In the fructose and glucose phases, subjects were given beverages equivalent to 25% of the subject's estimated energy requirement, and an iso-volumetric amount during the aspartame phase. Subjects were instructed to consume the entire beverage each day in four separate servings. In addition, subjects were provided with a standardized diet which they consumed ad libitum. The dietary intervention periods were separated by washout periods of 20 days.

Study A (n = 9): Fructose, Glucose, and Aspartame administered in random order



Study B (n = 24): Fructose, Glucose, and HFCS administered in random order

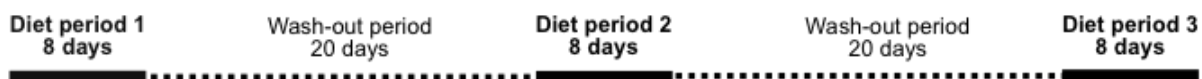


Figure 2.1. Study designs.

In both study A and B, participants completed each of three diet periods during which they consumed standardized solid foods *ad libitum* as well as 4 mandatory servings per day of beverages sweetened with fructose, glucose, or aspartame (study A) or fructose, glucose, or high-fructose corn syrup (HFCS) (study B). The order in which the beverages were consumed was randomized, and the intervention periods were separated by wash-out periods of 20 days each. All foods and beverages were provided, and *ad libitum* energy intake was assessed by weighing

all foods that were not consumed and subtracting those calories from the number of calories in the foods provided.

Study B was identical to study A except that a HFCS (55% fructose, 41% glucose, and 4% higher saccharides) phase was included in place of the aspartame phase. In both studies, aspartame was included in the glucose and HFCS beverages in order to match the sweetness of the fructose beverage. This was achieved by using a concentration of aspartame in the aspartame-, glucose-, and HFCS-sweetened beverages that, on average, received the same sweetness rating as the fructose-sweetened beverage by a panel of volunteers. The order in which subjects consumed the beverages was randomized and subjects, kitchen, and study staff were blinded as to which order subjects received the beverages. The randomization scheme was generated by the PI using block randomization, stratified for sex and adiposity group (normal weight vs. overweight/obese). A random number generator produced blocks of six numbers consisting of the numbers one through six in random orders. These blocks were then randomly grouped and ordered to generate randomization lists for the four strata defined by sex and adiposity group. Neither the study coordinators nor the PI were aware of the randomization code, and beverage preparation was conducted by individuals who did not communicate with participants or members of the study or kitchen teams in order to ensure blinding was maintained until the end of the study.

All foods were prepared and packaged specifically for each subject by the UW Nutrition Research Kitchen in Study A and the FHCRC Human Nutrition Laboratory in Study B. The solid food diet was designed as a 4-day rotating menu patterned after the average American diet (50% carbohydrate, 34% fat, and 16% protein) and was identical in all three phases of both studies. Subjects were provided solid food in an amount equal to 125% of their estimated daily calorie needs, as calculated by the Mifflin formula to determine their estimated resting energy expenditure, with an activity factor based on their habitual physical activity calculated from a modified Blair Physical Activity Questionnaire administered during the screening visit (113). In total, subjects were provided with 150% of their estimated energy requirements (25% mandatory consumption from the sweetened beverages and 125% ad libitum consumption from the standardized solid foods diet), except during the aspartame phase, in which they were provided

with 125% of their estimated energy requirements in the form of solid foods and 4% of their estimated energy requirements from the aspartame-sweetened beverage. Subjects were asked to eat from the solid foods provided until they were comfortably full and to return any uneaten foods to kitchen staff.

On day 1 of each intervention period, subjects were given detailed instructions regarding the diet. Subjects were advised to complete a checklist each day and record the foods consumed with an estimation of the uneaten portions to be returned. Subjects visited the research kitchens a minimum of 2 times per diet period to pick up food and drop off leftovers. Uneaten, returned foods were weighed and recorded, and compared to the subject's daily checklist for consistency. Any discrepancy between the checklist and the kitchen weigh-back was resolved with the subject immediately. In this manner, subject compliance with the diet and protocol was confirmed. Diets were designed using ProNutra software and weigh-back of returned food occurred using the ProNESSY software package for ProNutra (version 3.4.0.0; Viocare, Inc, Princeton, NJ). Uneaten food portions were weighed in their original containers and the net caloric intake was determined by subtraction for each day of each diet period. The overall total caloric intake was then calculated for each diet period. Subjects were also asked to record any non-study foods consumed during each diet period and calories from these foods were calculated as a measure of subject compliance.

2.2.2. Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences for Macintosh (versions 16.0 and 20.0; IBM Corporation). Distribution of variables was analyzed by checking histograms and normal plots of the data, and normality was tested by means of Kolmogorov-Smirnov and Shapiro-Wilk tests. Because Study A was designed to be a pilot study, no sample size calculation was conducted. Study B was powered to detect a clinically significant difference in markers of systemic inflammation between diet groups. The level of significance was set to $p < 0.05$ for all analyses. We conducted intent-to-treat and per protocol analyses, both of which yielded identical results in all regards. In the intent-to-treat analysis, we carried the last value forward for those time points for which no data were available. We therefore report only the results of the per protocol analyses because our primary interest is in the biological effects of the interventions.

2.2.2.1. Study A

A repeated measures analysis of variance (RM-ANOVA) was used to determine whether the within-subjects variable ‘diet’ explained any variation in body weight or the mean energy intake from solid foods or total energy during the three 8-day diet periods. A post hoc paired t-test with Bonferroni correction was used to compare caloric intakes between the fructose vs. glucose diet periods, the fructose vs. aspartame diet periods, the aspartame vs. glucose diet periods, and to compare energy intake during the fructose and glucose diet periods expressed as a percentage of energy intake during the aspartame diet period.

2.2.2.2. Study B

The primary analysis utilized RM-ANOVA to assess whether the within-subjects variable ‘diet’ explained any variation in mean overall energy intake, calories from solid foods, or physical activity. We also used RM-ANOVA to assess whether there were any changes in body weight and waist circumference between days 1 and 9 of each diet period, and whether this change differed by diet period. The data were then stratified by adiposity category and sex in the RM-ANOVA to assess whether energy intake was differentially affected by the three diet periods within these sub-groups. We also ran RM-ANOVA stratified by 4-day menu block to determine whether energy intake differed between the three diet periods during the first 4 days compared to the second 4 days of each diet period. All variables as well as residuals from RM-ANOVA were tested for normality by conducting a Shapiro-Wilk test and by checking histograms and normal plots. Variables were log(10)-transformed if they were not normally distributed. This was the case for body weight, waist circumference, overall energy intake, and energy intake within each 4-day diet block.

As sensitivity analyses, we further ran a series of multiple linear regression models to assess whether adjusting for diet order, physical activity (MET-h/week), subject self-reported minor illness (as a categorical variable defined as either 0 = healthy or 1 = ill), adiposity [in categories defined as 0 = normal weight (BMI < 25.0 kg/m²) and 1 = overweight or obese (BMI ≥ 25.0 kg/m²)], age, or sex affected the relationship between diet period and total energy intake (dependent variable). A final model included all variables the inclusion of which changed the β -coefficient describing the relationship between diet period and energy intake by more than 10%.

Because these analyses were conducted as sensitivity analyses, we did not adjust for multiple testing.

2.3. Results

2.3.1. Study A

Ten subjects were enrolled into the study. However, one female subject was excluded because of lack of compliance with the study protocol. All analyses were initially conducted as intent-to-treat with the inclusion of the one subject who failed to complete the entire study. The results did not differ whether this subject was included or excluded. Therefore, the *per protocol* analysis including only those participants who completed all study procedures is presented herein. Subject characteristics for the nine subjects who completed all study procedures per protocol between December 2009 and August 2011 are presented in Table 2.1. Overall, total energy intake was different among the three diet groups ($p < 0.001$ for diet effect in RM-ANOVA, Table 2.2), with statistically significantly greater energy intake in both the glucose and fructose phases ($p < 0.003$ for both sugars vs. aspartame in post hoc t-tests, Figure 2.2). However, we found no evidence of a difference in total energy intake between the fructose and glucose diet periods ($p = 0.462$ in post hoc t-test, Figure 2.2). We also found no evidence of a difference among groups in the amount of solid food calories consumed ($p = 0.224$ for diet effect in RM-ANOVA) (Table 2.2). There were no statistically significant differences in body weight between the three diet periods, with median (interquartile range) body weights of 66.1 (59.9, 69.1) kg, 66.5 (61.7, 69.5) kg, and 66.6 (59.7, 70.1) kg at the end of the fructose, glucose, and aspartame diet periods ($p = 0.243$).

Table 2.1. Characteristics of study participants at baseline¹.

	Study A (n=9)	Study B (n=24)		
	Normal weight	Normal weight	Overweight/obese	All
Sex (female/male)	5/4	3/9 ^a	6/6 ^a	9/15
Age (y)	21 ± 2.0	33 ± 11 ^a	39 ± 12 ^a	36 ± 12
BMI (kg/m ²)	22.7 ± 1.3	23.7 ± 1.0	31.0 ± 4.3	27.4 ± 4.8
Fasting glucose (mg/dL)	83 ± 6.9	87 ± 10 ^a	96 ± 8 ^b	92 ± 10
Physical activity (MET-h/wk)	44.2 ± 18.7	81 ± 54.5 ^a	56.3 ± 33.1 ^a	68.7 ± 45.9
Estimated total calorie requirement (kcal/d)	2,230 ± 398	2,610 ± 380 ^a	2,510 ± 370 ^a	2,560 ± 370

¹ Data are means ± SD. For study B, values in the same row with different superscript letters are significantly different from each other, $p < 0.05$ in *post hoc* independent samples t-tests. No statistical test was performed on BMI as the adiposity categories were designed to be different.

Table 2.2. Energy intake and diet composition from solid foods, sweetened beverages, and overall during each dietary period in Study A¹.

	Fructose (n=9)	Glucose (n=9)	Aspartame (n=9)	p-value (diet) ²
<u>Solid foods (<i>ad libitum</i>)</u>				
Energy intake (kcal/d)	2,180 ± 520	2,120 ± 595	2,220 ± 637	p = 0.224
Energy intake (% of estimated energy requirements)	97 ± 9.5	94 ± 12	99 ± 15	p = 0.213
Protein (% of total energy intake)	13.4 ± 0.73 ^a	13.8 ± 0.83 ^a	16.3 ± 0.50 ^b	p < 0.001
Fat (% of total energy intake)	24.4 ± 2.8 ^a	24.2 ± 2.7 ^a	29.3 ± 3.2 ^b	p < 0.001
Carbohydrate (% of total energy intake)	42.8 ± 2.1 ^a	42.2 ± 2.4 ^a	50.4 ± 2.9 ^b	p < 0.001
<u>Sweetened beverages (mandatory)</u>				
Energy intake (kcal/d)	515 ± 92	509 ± 92	85 ± 15 ³	
Fructose (% of total energy intake)	19.3 ± 1.6	0	0	
Glucose (% of total energy intake)	0	19.8 ± 2.1	0	
<u>Overall energy intake</u>				
Energy intake (kcal/d)	2,698 ± 607 ^a	2,629 ± 682 ^a	2,307 ± 651 ^b	p < 0.001
Energy intake (% of estimated energy requirement)	120 ± 9.6 ^a	117 ± 12 ^a	102 ± 15 ^b	p < 0.001
Energy intake (% relative to aspartame phase)	119 ± 11 ^a	115 ± 8.4 ^a	100 ± 0 ^b	p < 0.001

¹ Data are means \pm SD over each 8-day diet period.

² Reflects an overall comparison of the 3 dietary phases by RM-ANOVA. No statistical tests were performed on sweetened beverage intake as these were designed to be different.

³ Calories from beverages in the aspartame phase provided by carbohydrate fillers included in the sweetener.

Values in the same row with different superscript letters were significantly different from each other, $p < 0.05$ in *post hoc* paired t-tests.

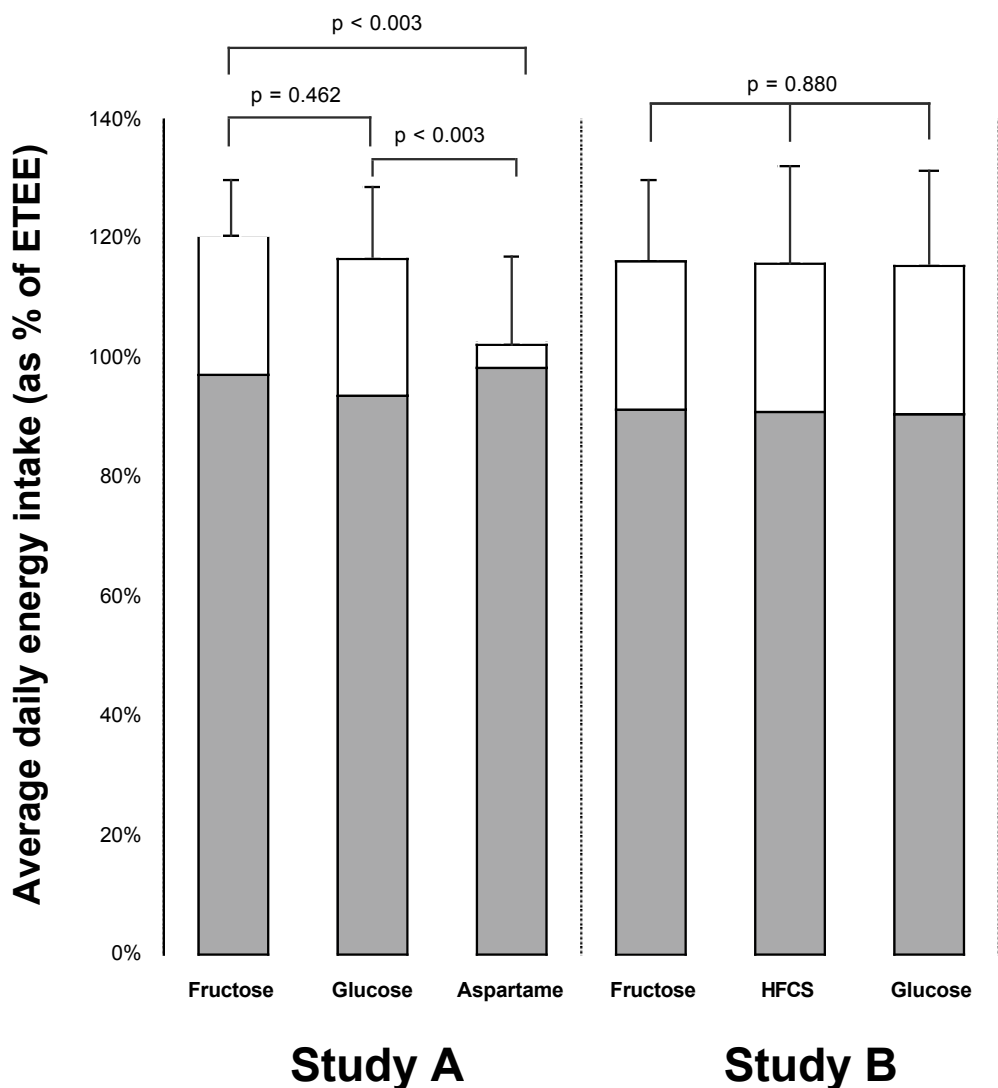


Figure 2.2. Mean (\pm SD) daily energy intake from solid foods (grey portion of the bars) and beverages (white portion of the bars), as a percentage of estimated total energy requirement during each diet period for study A (n=9) and study B (n=24).

Energy intake from solid foods was not affected by the calorie content of the sweetened beverages or the type of sugar used to sweeten beverages in either study, which led to lower overall energy intake in participants consuming aspartame-sweetened beverages compared to fructose- or glucose-sweetened beverages in study A. No evidence of a difference in total energy intake was observed when participants consumed beverages sweetened with fructose, high-fructose corn syrup (HFCS), or glucose. P-values were determined by *post hoc* paired t-tests with Bonferroni correction for multiple testing for study A, and by RM-ANOVA for study B.

2.3.2. Study B

To meet the predetermined sample size for the primary endpoint (n=24 to complete the study per protocol), twenty-five subjects were enrolled into the study. One subject was disenrolled after completion of her first diet period due to inability to complete the required clinic visits for the remaining diet periods. Analyses were initially conducted as intent-to-treat with the inclusion of all 25 subjects who initiated the study. Inclusion or exclusion of the disenrolled subject did not affect the results; therefore, only the per protocol analysis is presented herein. Subject characteristics for the 24 participants who completed all study procedures between January 2012 and April 2014 are shown in Table 2.1.

Compliance with study procedures and clinic visits was very good. Only nine of the 24 subjects consumed some form of solid food that was not provided by the research kitchen. On average, 0.08% of energy was consumed as non-study food items, ranging from 0.015 to 0.41% of subject's total calorie intake across all three diet periods (data not shown). There were no statistically significant changes in body weight or waist circumference within the 8-day diet periods, and no effect of diet group on either variable (Table 2.3). Physical activity also was not statistically significantly different among the three diet periods ($p=0.203$). Energy intake was not statistically significantly different among the three diet periods, whether expressed as total calorie intake or normalized to each subjects' estimated total calorie requirements (Table 2.4). Consistent with study A, subjects consumed on average 16% more energy than their estimated energy requirement predicted they would need (Table 2.4, Figure 2.2). We also found no evidence of a difference among the diet groups in the macronutrient composition of the solid foods consumed (Table 2.4).

Table 2.3. Changes in anthropometrics and physical activity during each diet period in Study B participants¹.

	Fructose (n=24)	HFCS (n=24)	Glucose (n=24)	p (time)²	p (time x diet)²
Body weight (kg)					
Day 1	78.6 (74.2, 85.9)	79.6 (74.6, 85.4)	79.5 (74.0, 87.3)	p = 0.142	p = 0.452
Day 9	78.0 (73.7, 86.0)	79.5 (74.0, 86.3)	79.3 (74.4, 86.3)		
Waist circumference (cm)					
Day 1	94.0 (87.0, 130.00)	91.5 (85.0, 101.5)	91.8 (86.5, 101.0)	p = 0.208	p = 0.346
Day 9	92.5 (86.3, 101.5)	91.8 (86.0, 103.5)	93.0 (83.8, 101.5)		
p (diet)²					
Physical activity (MET-h/wk)	56.1 (19.7, 68.6)	55.9 (23.2, 80.8)	50.2 (25.3, 87.3)	p = 0.203	

¹ Data are medians (IQR)

² Reflects an overall comparison of the 3 dietary phases by RM-ANOVA.

Table 2.4. Energy intake and diet composition from solid foods, sweetened beverages, and overall during each dietary period in Study B¹.

	Fructose (n=24)	HFCS (n=24)	Glucose (n=24)	p-value (diet)²
<u>Solid foods (<i>ad libitum</i>)</u>				
Energy intake (kcal/d)	2,330 ± 421	2,310 ± 482	2,300 ± 409	p = 0.836
Energy intake (% of estimated energy requirements)	92 ± 14	91 ± 16	91 ± 16	p = 0.925
Protein (% of total energy intake)	14.3 ± 0.64	14.4 ± 0.74	14.3 ± 0.57	p = 0.838
Fat (% of total energy intake)	26.7 ± 2.8	26.6 ± 3.2	26.7 ± 3.3	p = 0.980
Carbohydrate (% of total energy intake)	38 ± 3.1	38 ± 3.6	38 ± 2.9	p = 0.699
<u>Sweetened beverages (mandatory)</u>				
Energy intake (kcal/d)	638 ± 92	640 ± 91	641 ± 93	
Fructose (% of total energy intake)	21.6 ± 2.3	12.1 ± 1.7	0	
Glucose (% of total energy intake)	0	9.9 ± 1.4	22.0 ± 3.1	
<u>Overall energy intake</u>				
Energy intake (kcal/d)	2,970 ± 482	2,950 ± 535	2,940 ± 460	p = 0.880
Energy intake (% of estimated energy requirement)	116 ± 13.6	116 ± 16.2	116 ± 15.9	p = 0.880

¹ Data are means ± SD over each 8-day diet period.

² Reflects an overall comparison of the 3 dietary phases by RM-ANOVA. No statistical tests were performed on sweetened beverage intake as these were designed to be different.

Secondary analyses stratified by sex and adiposity found no evidence of a differential impact of the diets on energy intake in men vs. women or in normal weight vs. overweight/obese individuals (data not shown). We also ran sensitivity analyses excluding six subjects who reported a minor illness (such as a cold) during one or more of the diet periods, which did not change the result (data not shown). As another sensitivity analysis, we re-ran all analyses with the exclusion of 10 subjects whose total energy intake varied by more than 10% of their estimated energy requirement between any two diet periods and confirmed that the exclusion of these subjects also did not change the overall finding (data not shown).

Multiple linear regression analyses were conducted as further sensitivity analyses. These analyses confirmed that overall energy intake was not associated with the type of sugar used to sweeten the beverages, and that adjustment for diet order, physical activity, illness, adiposity, age, and sex did not materially change that relationship (data not shown).

Because it may be possible that energy homeostatic compensatory mechanisms attenuate any effect of the fructose content of SSBs on energy intake within a few days, we also assessed whether energy intake differed between the three dietary periods just in the first 4, or just in the last 4 days of each diet period. As for the entire 8-day period, we did not detect even a trend for a difference in energy intake among the three dietary phases within each of these 4-day periods (data not shown). And lastly, we tested whether there were any differences in calorie intake between the first and second 4-day diet blocks within each 8-day period. Overall energy intake was statistically significantly greater by 87 ± 507 kcal during the first 4 days of the diet periods compared to the second 4 days ($p = 0.003$ for the difference between the first vs. second 4-day block in RM-ANOVA). We found no evidence that the reduction in energy intake between the first and the second 4-day diet block differed among the three dietary periods (time x diet interaction $p=0.228$). No unexpected adverse events or adverse events that were more severe than ‘mild’ occurred in either study.

2.4. Discussion

The major finding from study A was that healthy, normal weight adults consumed statistically significantly more calories when 25% of their estimated energy requirement was provided as a glucose- or fructose-sweetened beverage, as compared to an iso-volumetric amount of an

aspartame-sweetened beverage. On average, this caloric excess was approximately 17%, demonstrating that participants compensated only for ~1/3 of the calories consumed in the SSB. This finding was supported by study B in that normal and overweight/obese subjects also consistently consumed 16% more energy during each diet phase than their estimated energy requirement predicted they would need to maintain energy homeostasis. The fructose-to-glucose ratio of the beverages did not influence overall energy intake.

Our results are in line with observational studies that consistently show that consumption of SSBs promotes weight gain in children and adults (12). Although our studies did not compare liquid vs. solid sugar calories, our findings are also consistent with previous data demonstrating failure to fully compensate for liquid sugar calories. For example, DiMeglio and Mattes (72) conducted a crossover study in which healthy subjects consumed 450 kcal in the form of either jelly beans or soda in addition to their *ad libitum* habitual diet for 4 weeks each. During the solid sugar phase, subjects completely compensated for the calories consumed as jelly beans by reducing their intake of solid foods. In contrast, during the soda phase, subjects failed to decrease their intake of solid foods, which remained at a level equivalent to their habitual intake. Body weight increased to a statistically significant degree during the liquid calorie phase only (72).

A remaining open question was whether differing adipogenic effects are exerted by the glucose and fructose moieties that make up most caloric sweeteners. Meta-analyses showed no evidence that fructose differentially affected weight when isocalorically exchanged for glucose in energy-matched diets (87). Arguably more relevant, however, is whether calorically matched fructose vs. glucose may exert a differential effect on overall *ad libitum* energy intake. Our studies sought to answer this question by making the consumption of varying ratios of fructose vs. glucose mandatory while allowing subjects to eat from a standardized diet *ad libitum*. Stanhope, et al. (81) instructed overweight subjects to consume 25% of their estimated energy intake in the form of a fructose- vs. glucose-sweetened beverage while also consuming their habitual diet for 10 weeks. Subjects in both arms gained a similar amount of weight. This result supports our conclusion that we found no evidence that fructose-sweetened beverages differentially affect energy intake compared to glucose-sweetened beverages. However, this study was not randomized, and our studies offer an additional level of confidence because of the strong randomized cross-over design, provision of all foods for the entirety of the study periods, and the

fact that we included a fructose malabsorption screening test which we suggest is critical for true assessment of the effect of 100% fructose-sweetened SSBs on energy intake.

Our studies were specifically designed to be medium-term in length to prevent statistically significant weight gain by subjects over the course of the intervention. This avoided confounding of the relationship between sweetened beverage type and energy intake by substantial changes in adiposity during the experimental diet periods. There are a number of reasons why we may not have detected even a small increase in weight. First, the study was not designed or powered to detect changes in weight. Second, it is likely that subjects lost some body water during the study diet periods, which we frequently see in the first few days after subjects switch to a controlled study diet with lower sodium content. Thus, it is important not to interpret the lack of weight gain in these moderate-term diet periods as evidence that weight would remain stable in individuals consuming SSBs over longer periods of time.

We believe that the cross-over design was a major strength of our study, as it allowed us to compare energy consumption within each person on each diet; however, the relatively short duration of each intervention period is also a possible limitation. Theoretically, it is possible that differences could become apparent if these SSBs were consumed for more than 8 days. However, substantial differences in key appetite and satiety-regulating hormones including insulin, leptin, and ghrelin, are apparent within the first day of consuming a similar amount of fructose- vs. glucose-sweetened beverages (76). It appears implausible that acute hormonal differences would lead to differences in *ad libitum* energy intake only more than 8 days later. Because not even the slightest trend was apparent in our studies, we are confident that the differences in the response of satiety-regulating hormones between fructose- and glucose-sweetened beverages described in previous studies do not translate into differences in energy intake. We did observe a statistically significant decrease in the overall energy consumed during the second 4 days on the diet compared to the first 4-days. This suggests that energy homeostatic mechanisms are engaged to some degree over an 8-day period and that our methodology was sufficiently precise to detect this effect. However, the difference in energy intake during the first vs. the second 4-day periods was independent of the fructose content of the beverages. Failure of the fructose content of the SSB to affect this adaptive response to caloric excess further strengthens our central conclusion. We also stratified by sex and adiposity and did not detect a difference in energy intake according

to beverage type within these sub-groups. While the sample sizes in these sub-group analyses were relatively small and lack of power could be a concern, energy intake was strikingly similar across all diet phases and not even a trend towards a difference could be detected.

Another potential limitation is that the total number of calories provided to participants in study A differed across study arms, as participants received 150% of their estimated total energy requirement in the fructose and glucose arms (125% from solid foods, 25% from SSBs), but only 129% in the aspartame arm (125% from solid foods, 4% from the aspartame-sweetened beverage). This may have contributed to the greater total energy intake in the fructose and glucose arms. However, this design was necessary to conduct this trial in a double-blinded fashion, as providing more solid food in one arm would have partially un-blinded participants. There is also a rich literature suggesting that total energy intake is affected by the total volume rather than the total energy content of the food served (114-116). We therefore felt that providing more solid (i.e., visible) food to subjects, and therefore more overall volume, would have been very likely to increase total energy intake in the aspartame arm.

Our findings are strengthened by the fact that we obtained essentially the same result in these paired studies. Another strength of our study was the provision of all foods, which allowed us to accurately assess energy intake and experimentally control other dietary factors that may affect *ad libitum* energy intake. However, by providing subjects with all foods, we left little opportunity for subjects to self-select food items. Because the study diet did not include highly palatable snack foods high in sugar and fat, we could not test whether the beverages tested here affect the types of solid foods participants would eat if they had the ability to choose, which in return could affect total energy intake.

In conclusion, these studies strengthen the argument that SSBs promote an increase in overall energy intake in healthy adults over the medium term, which over longer periods of time would be expected to result in weight gain. Furthermore, our data provide no evidence that fructose and glucose differ in terms of energy intake regulation when consumed in the form of a SSB. Rather, our study shows that both glucose and fructose consumed in liquid form promote excess energy intake by failing to invoke a concomitant reduction of energy from solid foods.

3. NO EVIDENCE OF A DIFFERENTIAL EFFECT OF BEVERAGES SWEETENED WITH FRUCTOSE, HIGH-FRUCTOSE CORN SYRUP, OR GLUCOSE ON SYSTEMIC OR ADIPOSE TISSUE INFLAMMATION IN NORMAL WEIGHT TO OBESE ADULTS: A RANDOMIZED CONTROLLED TRIAL

Sugar-sweetened beverage (SSB) consumption and low-grade, chronic inflammation are both independently associated with type 2 diabetes and cardiovascular disease. Fructose, a major component of SSBs, may acutely trigger inflammation, which may be one link between SSB consumption and cardiometabolic disease. The primary aim of the study was to determine whether fructose- vs. high-fructose corn syrup (HFCS)- vs. glucose-sweetened beverages differentially influence systemic inflammation (fasting plasma C-reactive protein and interleukin-6, primary endpoint) acutely and prior to major changes in body weight. Secondary endpoints included adipose tissue inflammation as well as intestinal permeability and plasma fetuin-A as potential mechanistic links between fructose intake and low-grade inflammation. We conducted a randomized, controlled, double-blind cross-over design dietary intervention (the Diet and Systemic Inflammation [DASI] study) in 24 normal weight to obese adults without fructose malabsorption. Participants drank 4 servings/d of a fructose-, glucose-, or HFCS-sweetened beverage comprising 25% of estimated calorie requirements while consuming a standardized diet *ad libitum* for three, 8-d periods. Subjects consumed 116% of their estimated calorie requirement while drinking the beverages, with no evidence of a difference in total energy intake or body weight among groups, as reported previously. Fasting plasma concentrations of CRP ($p=0.457$) and IL-6 ($p=0.933$) did not differ to a statistically significant degree at the end of the 3 diet periods. We did not detect a consistent differential effect of the diets on measures of adipose tissue inflammation, except for adiponectin gene expression in adipose tissue ($p=0.005$), which was lowest following the glucose phase. We also did not detect consistent evidence for a differential impact of these sugars on measures of intestinal permeability (lactulose-mannitol test, plasma zonulin, and plasma lipopolysaccharide-binding protein). Our data show no evidence of a differential effect of excessive amounts of fructose vs. HFCS vs. glucose from SSBs consumed over 8 days on low-grade chronic systemic inflammation in normal weight to obese adults.

3.1. Introduction

The consumption of sugar-sweetened beverages (SSBs) is associated with type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (22, 32, 34) both independently and through the increased risk conferred by excess body fat. Chronic, low-grade inflammation is associated with both of these diseases and is also highly associated with obesity. Thus, the association between SSB consumption and the increased risk of chronic disease may be partly mediated by low-grade, chronic inflammation.

Numerous lines of evidence from both animal and human studies suggest that the fructose component of the SSB may be largely responsible for the observed associations between SSB intake and risk factors for metabolic disease such as low-grade chronic inflammation (37, 99, 100, 117), dyslipidemia (89), and decreased insulin sensitivity (81). We hypothesized that fructose-, but not glucose-sweetened beverages, may acutely trigger low-grade chronic inflammation in the systemic circulation as well as in adipose tissue, in a manner independent of weight change through one of two different mechanisms. First, high fructose consumption, even under short-term, iso-caloric conditions, promotes translocation of microbial material from the gut lumen to the portal circulation in rodents, thereby triggering low-grade hepatic and systemic inflammation (98, 99). As systemic inflammation can trigger the activation of inflammatory pathways in adipose tissue (118), we hypothesized that hepatic and systemic inflammation triggered by increased intestinal permeability and endotoxemia may also result in changes in adipose tissue inflammation. Second, even under isocaloric conditions, substituting liquid fructose in place of solid carbohydrate foods results in statistically significantly increased *de novo* lipogenesis (DNL) in the liver within just 9 days (90). DNL is thought to be a major determinant of fetuin-A production by the liver (92, 119), and fetuin-A in return has been hypothesized to be a chaperone for free fatty acids (FFA) to activate inflammatory pathways in adipose tissue macrophages (120). Thus, it is plausible that fructose-induced DNL may lead to an increase in plasma fetuin-A concentrations thereby triggering an increase in low-grade chronic inflammation in adipose tissue as well as systemically.

We previously reported the results from our controlled, crossover design dietary intervention study entitled ‘Diet and Systemic Inflammation’ (DASI) on *ad libitum* energy intake (121). The present manuscript describes the second set of analyses from this study in which we evaluate

whether 8-d consumption of high amounts of fructose in the form of SSBs promotes low-grade chronic systemic and adipose tissue inflammation in normal weight to obese adults. We further sought to determine whether this high dose of fructose in the form of SSBs affects intestinal permeability and plasma fetuin-A concentrations, both of which could be mechanisms linking excessive fructose intake to low-grade chronic inflammation in adipose tissue and the systemic circulation.

3.2. Methods

3.2.1. Subjects

The DASI study was carried out at the Fred Hutchinson Cancer Research Center (FHCRC) in Seattle, WA, between December 2011 and April 2014. A detailed description of the study has been published previously (121). Twenty-five men and women [between 20 and 40 kg/m², age 18-65 y] were recruited by newspaper advertisements and fliers posted in the Seattle area. Subjects were recruited into 2 categories based on BMI: normal weight (BMI: 20.0-24.9; n = 13) and overweight/obese (BMI: 25.0-39.9; n = 12).

Subjects were required to have been weight stable to within 4.5 kg (10 pounds) of their lifetime maximum weight and had to be willing to consume only food and beverages provided by the research kitchen for 3 periods of 8 d each. Potential subjects were required to be available for admission to the clinic on up to 6 occasions. Other exclusion criteria included smoking or the use of recreational drugs; alcohol abuse (>2 drinks/d); a history of cardiovascular disease or diabetes mellitus; the presence or history of any chronic inflammatory, autoimmune, or metabolic disease; recent (within 12 mo) pregnancy or breastfeeding; the presence of phenylketonuria, hereditary fructose intolerance, or other malabsorption syndromes; the presence or recent (within 2 mo) history of anemia; and the current or recent (within 3 mo) use of insulin, antidiabetics, b-blockers, glucocorticoids, anabolic steroids, warfarin, antibiotics, probiotics, or nonsteroidal anti-inflammatory drugs (daily use). Before being enrolled, subjects underwent telephone and in-person screening interviews to assess medical history, obtain a plasma biochemistry panel, and conduct a fructose malabsorption test (111) to assess eligibility. Briefly, during the in-person screening visit, each subject consumed a beverage sweetened with fructose in an amount equivalent to 6.25% of estimated daily calorie intake (representing 1 serving of the study beverage) as estimated by the Mifflin formula (112) and a standardized activity factor of 1.5. The

hydrogen content of the exhaled breath was measured at baseline and 0, 30, 60, 90, 120, 150, and 180 min after consumption of the beverage. An increase of >20 ppm above baseline for 2 subsequent time points was considered indicative of fructose malabsorption, for which a subject was excluded from the study. Written informed consent was obtained from all subjects, and the study was approved by Fred Hutchinson Cancer Research Center institutional review board.

3.2.2. Study design, diets, and clinic visits

The primary aim of the DASI study was to assess whether glucose- vs. HFCS- vs. fructose-sweetened beverages differentially influence markers of systemic inflammation, as assessed by measuring the fasting plasma concentrations of CRP and IL-6. *Ad libitum* energy intake was a pre-determined secondary endpoint of this study along with fasting plasma adiponectin, zonulin, and lipopolysaccharide-binding protein (LBP), urinary lactulose/mannitol ratio, and adipose tissue inflammation. Results of the energy intake endpoint have been published previously (121).

The study design is presented in Figure 3.1. On day 1 of each intervention period, subjects were admitted to the FHCRC prevention center after a 10-h fast. After ruling out pregnancy in women of child-bearing potential, anthropometrics and vital signs were recorded. A health questionnaire was administered to determine if subjects had been sick over the previous 20-d period. Blood was then collected by venous puncture into chilled EDTA-tubes, spun immediately at 3,000 g for 10 min, aliquoted, and stored at -70°C.

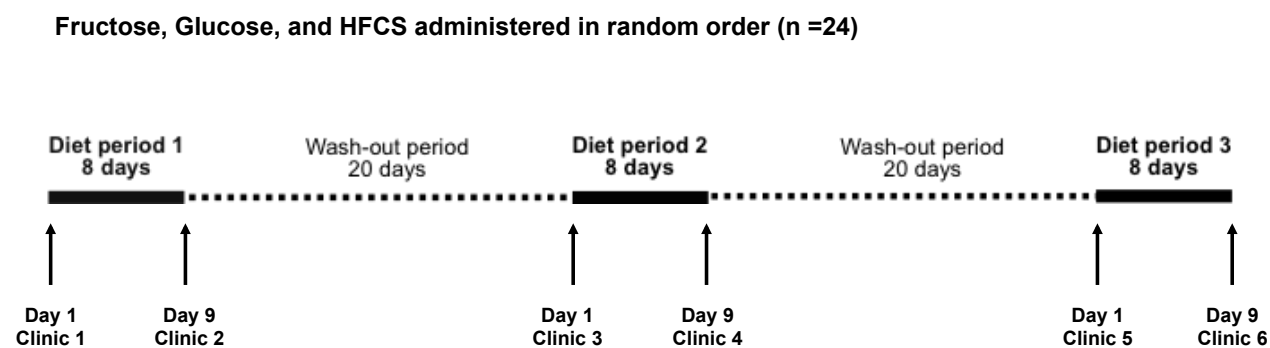


Figure 3.1. Study design.

Participants completed each of three diet periods during which they consumed standardized solid foods *ad libitum* as well as 4 mandatory servings per day of beverages sweetened with fructose, glucose, or HFCS. The order in which the beverages were consumed was randomized, and the intervention periods were separated by washout periods of 20 d each. All foods and beverages

were provided, and *ad libitum* energy intake was assessed by weighing all foods that were not consumed and subtracting those calories from the number of calories in the foods provided. HFCS, high-fructose corn syrup.

Following the clinical procedures, subjects were provided with detailed instructions regarding the diet: For a period of 8 days, subjects were required to consume 25% of their estimated daily calorie needs in the form of a beverage sweetened with either 100% fructose, 100% HFCS (55% fructose, 41% glucose, and 4% higher saccharides), or 100% glucose. Aspartame was included in the glucose and HFCS beverages to match the sweetness of the fructose beverage. This was achieved with the use of a concentration of aspartame in the glucose- and HFCS-sweetened beverages that, on average, received the same sweetness rating as the fructose-sweetened beverage by a panel of volunteers. The order in which subjects consumed the beverages was randomized, and subjects, kitchen, and all study staff were blinded as to which order subjects received the beverages. The randomization scheme was generated by the principal investigator with the use of block randomization, stratified for sex and adiposity group (normal weight compared with overweight/obese). A random number generator produced blocks of 6 numbers consisting of the numbers 1–6 in random orders. These blocks were then randomly grouped and ordered to generate randomization lists for the 4 strata defined by sex and adiposity group. Neither the study coordinators, the principal investigator, the laboratory staff carrying out the outcome assessments, nor the statistician were aware of the randomization code, and beverage preparation was conducted by individuals who did not communicate with participants or members of the study or kitchen teams to ensure blinding was maintained until the end of the study (121).

In addition to the beverages, subjects were provided with 125% of their estimated daily calorie needs as a standardized diet which they consumed *ad libitum*. In total, subjects were provided with 150% of their estimated energy needs, with 25% mandatory consumption as the SSB, and 125% provided as solid foods to be consumed *ad libitum*. All foods were prepared and packaged specifically for each subject by the Fred Hutchinson Cancer Research Center Human Nutrition Laboratory. The solid food diet was designed as a 4-d rotating menu patterned after the average US diet (50% carbohydrate, 34% fat, and 16% protein) and was identical in all 3 phases of the

study. Subjects were provided solid food in an amount equal to 125% of their estimated daily calorie needs, as calculated by the Mifflin formula to determine their estimated resting energy expenditure, with an activity factor based on their habitual physical activity calculated from a modified Blair Physical Activity Questionnaire administered during the screening visit (113). In total, subjects were provided with 150% of their estimated energy requirements (25% mandatory consumption from the sweetened beverages and 125% *ad libitum* consumption from the standardized solid foods diet). Subjects were asked to eat from the provided solid foods until they were comfortably full and to return any uneaten foods to kitchen staff for weigh-backs. Subjects were required to complete a daily checklist of foods consumed with an estimation of the uneaten portions to be returned. Any discrepancy between the checklist and the kitchen weigh-back was resolved with the subject immediately, as subjects visited the research kitchen a minimum of 2 times per diet period to pick up food and drop off leftovers. As such, subject compliance with the diet and protocol was confirmed.

On day 9 of each intervention, clinic procedures identical to the day 1 visit were performed, with the addition of a modified Blair Physical Activity Questionnaire and a lactulose/mannitol test to assess intestinal permeability. For this test, fasted subjects were asked to void their bladders and then consume one final serving of the beverage they had received for the previous 8 days. In addition to the specific sugar (fructose vs. HFCS vs. glucose), the beverage also contained 5 g of lactulose and 2 g of mannitol. Urine was collected for the subsequent 5 hours. The urine was mixed, the total volume recorded, and aliquots were frozen at -70°C. The amount of each sugar recovered from the initial dose was used to calculate the lactulose/mannitol ratio and the percentage of lactulose recovered from urine. This is the most frequently used clinical method to assess intestinal permeability as these sugar probes are passively absorbed, are not metabolized, and pass into the urine unchanged (122).

All subjects were invited to undergo a voluntary adipose tissue biopsy procedure as an ancillary component of the larger study. Fourteen subjects elected to participate in the procedure, which has been described previously (123). Briefly, after local injection of a 2% lidocaine in saline solution, a small incision was made lateral to the umbilicus. An additional lidocaine solution was injected under the surface and a 14-gauge needle was used to extract approximately 1-2 g of adipose tissue. A small amount of tissue (three pieces of ~100 mg each) was frozen immediately

on dry ice and stored at -70°C for gene expression analysis. The remainder was used to quantify and characterize the tissue leukocytes by flow cytometry, as discussed in detail below.

3.2.3. Outcomes

The primary endpoints of this trial were fasting plasma concentrations of CRP and IL-6, both biomarkers of systemic inflammation. The secondary endpoints included measures of intestinal permeability (lactulose-mannitol-ratio and lactulose recovery based on a urinary lactulose-mannitol test; fasting plasma zonulin, and fasting plasma LBP, all interpreted together); measures of adipose tissue inflammation (whole adipose tissue gene expression of adiponectin, tumor necrosis factor alpha (TNF- α), IL-1 β , IL-6, IL-10, chemokine (C-C motif) ligand (CCL2), and interferon gamma (IFN- γ) and adipose tissue content of macrophages, CD11c⁺ macrophages, neutrophils, dendritic cells, CD4⁺ T-cells, CD8⁺ T-cells, and the adipose tissue CD4⁺ to CD8⁺ T-cell ratio, all interpreted together); and fasting plasma adiponectin. Secondary endpoints that were not defined *a priori* included fasting plasma fetuin-A and FFA concentrations. An additional secondary endpoint was *ad libitum* energy intake during the three study diets, the results of which we published previously (121).

3.2.4. Plasma, urine, and adipose tissue analyses

The concentrations of plasma IL-6 and fetuin-A (both R&D Systems, Minneapolis, MN), adiponectin and zonulin (both ALPCO, Salem, NH), and LBP (Cell Sciences, Canton, MA) were measured by ELISA on day 9 samples. These assays were performed in triplicate, with all samples from one participant run on the same plate. Intra- and inter-assay coefficients of variation within our lab for these assays were 7.8% and 13.7% for IL-6, 9.2% and 8.8% for fetuin-A, 5.3% and 6.5% for adiponectin, 1.9% and 9.2% for zonulin, and 8.3% and 32.2% for LBP, respectively. Because of the high inter-assay variability for LBP, we normalized across plates using internal standards that were run twice in triplicate on each plate.

Measurements of high sensitivity CRP (immunonephelometry) and FFA (colorimetric micro-determination) were completed by Northwest Lipid Laboratories, Seattle, WA. Lactulose and mannitol concentrations in 5-h urine samples were measured by gas chromatography (columns supplied by Agilent Technologies, Santa Clara, CA) in the FHCRC Biomarker laboratory using a previously published method (124). Briefly, pooled urine from healthy volunteers was spiked

with lactulose and mannitol to create a 6-level standard curve with concentrations ranging from 0.02 to 5.00 mg/mL. The average recovery rate was 96.0% when pure sugar standards were added to pooled urine in this manner and the intra- and inter-assay coefficients of variation in the biomarker lab were 4.2% and 5.6%, respectively.

3.2.4.1. Collagenase digestion of adipose tissue

Adipose tissue was processed as described previously (125). Briefly, Collagenase I (Worthington Biochemical Corp., Lakewood, NJ; final concentration 0.5 mg/mL) in PBS with 50 U/mL DNase I was used to digest the tissue for 60 min at 37°C. Following digestion, the tissue slurry was passed through a 180 µm mesh filter and rinsed twice with 5 mL PBS + 1% BSA before centrifugation for 5 min at 1,200 rpm. The cell pellet containing the stromavascular cell fraction (SVC) was then resuspended in 1x red cell lysis solution (BD Pharm Lyse™, San Jose, CA) and incubated for 2 min at room temperature. After washing twice with cold staining buffer (0.2%BSA/0.09%NaN₃/PBS), the cell pellet was resuspended in 200 µL of staining buffer.

3.2.4.2. Immunophenotyping of adipose tissue stromavascular cells by flow cytometry

Multi-parameter flow cytometry was performed on the SVC fraction freshly isolated from adipose tissue. Stromavascular cells were labeled using a combination of up to 9 directly conjugated primary antibodies all purchased from either BD Pharmagen (San Jose, CA), BioLegend (San Diego, CA), or Beckman-Coulter (Brea, CA). They included Allophycocyanin (APC)-conjugated CD4 and CD206; APC-Cy7 conjugated CD3 and CD14; Alexa Fluor 700 conjugated CD8 and CD11c; Fluorescein Isothiocyanate (FITC)-conjugated CD40 and CD80; Krome Orange conjugated CD45; Peridinin Chlorophyll Protein Complex (PerCP)-Cy5.5 conjugated CD15 and CD20; Phycoerythrin (PE)-conjugated CD152 and CD163; PE-Cy7 conjugated CD1c; and PE-Texas Red conjugated CD16 and CD45RA. Samples were analyzed immediately following labeling using a LSRII flow cytometer (Beckton Dickinson, Franklin Lakes, NJ) to collect at least 30,000 events in a broadly defined lymphocyte-monocyte-granulocyte gate, defined by forward- and side-scatter attributes. Analyses were carried out with FlowJo software, version 9.4.1 (TreeStar, Ashland, OR) using histogram and dot plot analyses on the live cell gate. Live cells were defined by the absence of fluorescence associated with the uptake of 4',6-diamidino-2-phenylindole, 2HCl (DAPI; EMD Chemicals, Gibbstown, NJ and/or Molecular Probes, Invitrogen, Carlsbad, CA), a reactive dye that binds to cellular amines of

membrane-compromised (i.e. dead) cells. The expression of various combinations of these markers was used to identify and quantify specific sub-populations of leukocytes.

3.2.4.3. Gene expression analyses

Total RNA was extracted from whole adipose tissue using RNeasy Lipid Tissue kit (Qiagen, Hilden, Germany) and quantified using RiboGreen (Invitrogen Corp., Carlsbad, CA). cDNA synthesis was carried out on ~1 mg of total RNA using the RETROscript® Kit (Ambion/Applied Biosystems, Austin, TX) and PCR performed using pre-designed TaqMan® Gene Expression Assays (Applied Biosystems, Austin, TX) on an ABI Prism® 7900HT Sequence Detection System. Gene targets included adiponectin, IL-6, IL-10, TNF- α , CCL2, IL-1 β , and IFN- γ . We measured β -glucuronidase (GUSB) as a housekeeping gene as this is relatively stably expressed in adipose tissue across different conditions (126). A normalization factor was then calculated using this housekeeping gene and applied to all target genes.

3.2.5. Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences for Macintosh (version 20.0; IBM, Armonk, NY). The study was powered to detect clinically relevant differences among diet groups in the primary endpoints, CRP and IL-6. In a pilot study (study A in (121)), fasting plasma CRP and IL-6 concentrations were 0.92 ± 0.64 mg/L and 0.60 ± 0.40 pg/mL, respectively, higher after subjects had consumed 25% of their estimated calorie requirements in the form of a fructose-sweetened beverage compared to when they had consumed a glucose-sweetened beverage (n=10). Accordingly, it was determined that if 24 subjects completed the DASI study, we would have 80% power to detect a difference between any two groups of 0.41 mg/L for CRP and 0.26 ng/L for IL-6, assuming the same variability in response seen in the pilot study and an α -error level of 2.5% (adjusted to account for the two primary endpoint measures). We powered the study to detect smaller differences in both endpoints because smaller differences than those observed would still be clinically relevant, the variability in response may be greater than that observed in the pilot study, and to improve our power for subgroup analyses (normal weight vs. overweight/obese individuals). The level of significance was set to $p < 0.05$ for all analyses, except for CRP and IL-6, for which the level of significance was set to $p < 0.025$ as described above. Secondary endpoint measures were not adjusted for multiple testing, as all were related to the primary outcomes and adjustment for the

numerous outcomes was judged to overly inflate the β -error, i.e. reduce power to detect effects of the diets. The different outcomes related to intestinal permeability and adipose tissue inflammation are different measures of these biological processes, and are being considered, interpreted, and reported together (127).

The primary analysis utilized repeated measures analysis of variance (RM-ANOVA) to assess whether the within-subjects variable 'diet' explained any variation in mean plasma CRP or IL-6 measured on day 9 of each diet period. Similarly, this analysis was repeated on the remaining biomarkers of systemic and adipose tissue inflammation and intestinal permeability: LBP, adiponectin, zonulin, fetuin-A, FFA, the urinary lactulose/mannitol ratio as well as adipose tissue mRNA and immune cell populations which were also measured on day 9 of each diet period. We used RM-ANOVA to assess whether there were any changes in body weight or CRP between days 1 and 9 of each diet period, and whether this change differed by diet period. A paired t-test with Bonferroni adjustment was conducted *post hoc* to determine which two groups differed from each other if the RM-ANOVA indicated an overall difference. The data were then stratified by adiposity category in the RM-ANOVA to assess whether biomarkers of systemic inflammation and intestinal permeability were differentially affected by the three diet periods within these sub-groups. Stratified analyses were not conducted on biomarkers of adipose tissue inflammation as the number within subgroups was too small. All variables as well as residuals from RM-ANOVA were tested for normality by conducting a Shapiro-Wilk test and by checking histograms and normal plots. Variables were $\log(10)$ -transformed if they were not normally distributed. This was the case for CRP, IL-6, LBP, FFA, fetuin-A, the lactulose/mannitol ratio, the percent of lactulose and mannitol recovered from urine, and IL-1b, IL-6, IL-10, CCL2, and IFN γ mRNA as well as neutrophil, dendritic cell, 11c⁺ macrophage, and the ratio of CD4⁺ to CD8⁺ T cell populations in adipose tissue.

For all endpoints, we ran follow-up sensitivity analyses excluding subjects who reported minor illness (such as a cold) during one or more of the diet periods to assess whether the relationship between diet and any of the measured biomarkers were affected by inclusion of these subjects.

Our *a priori* statistical analysis plan was a per-protocol analysis. Upon completion of the study, we conducted all analyses first as intent-to-treat and included all 25 subjects who initiated the study, with the last observation carried forward for the missing data points. The analysis was

then repeated per-protocol with the exclusion of the subject (n=24). This exclusion did not alter the results in any way, therefore only the results of the per-protocol analyses are presented herein.

3.3. Results

We conducted in-person screening visits in 63 individuals. Thirty-five participants did not meet eligibility criteria, of which 23 suffered from fructose malabsorption, while three individuals declined participation in the study. Twenty-five participants were enrolled, starting in January 2012, with the final subject completing the study in April 2014. One subject was disenrolled after completion of the first diet period for failing to complete the second and third diet period. The detailed baseline characteristics for the 24 participants who completed all study procedures have been described (121). Briefly, the 24 subjects were either normal weight (n=12; 3 females, 9 males) with a mean \pm SD age of 33 ± 11 y, BMI of 23.7 ± 1.0 kg/m², fasting glucose of 87 ± 10 mg/dL and baseline CRP of 1.1 ± 1.0 mg/L or overweight/obese (n=12; 6 females, 6 males) with a mean \pm SD age of 39 ± 12 y, BMI of 31.0 ± 4.3 kg/m², fasting glucose of 96 ± 8 mg/dL and baseline CRP of 2.5 ± 1.8 mg/L (121).

A detailed description of the effect of fructose- vs. HFCS- vs. glucose-sweetened beverages on overall energy intake has been published (121). In summary, subjects consistently consumed 16% greater total calories than their estimated energy requirement predicted they would need when 25% of their energy was consumed in the form of a sweetened beverage; however, we found no evidence that overall energy intake differed to a statistically significant degree among the three diet periods (121). In spite of the excessive energy intake, body weight did not change within the 8-d diet periods to a statistically significant degree and no statistically significant difference in weight change was seen among diet periods. Physical activity also was not statistically significantly different among the three diet periods (121).

3.3.1. Biomarkers of systemic inflammation and intestinal permeability

For our primary outcome measures, we found no evidence of an effect of diet on day 9 plasma concentrations of IL-6 (Table 3.1), nor was there a statistically significant change in CRP within the diet periods ($p=0.353$ for time in RM-ANOVA) or evidence of a difference in the change among diet periods ($p=0.403$ for time x diet group interaction in RM-ANOVA).

Table 3.1. Biomarkers of systemic inflammation in all subjects on day 9 of each dietary period, unless otherwise noted¹.

	Fructose (n=24)	HFCS (n=24)	Glucose (n=24)	p (time)²	p (diet)²	p (time x diet)²
CRP (mg/L)						
Day 1	0.91 (0.45, 2.35)	1.18 (0.38, 2.49)	1.67 (0.36, 2.91)	0.353	0.933	0.403
Day 9	1.07 (0.48, 2.10)	0.84 (0.49, 2.48)	1.09 (0.38, 3.04)			
IL-6 (pg/mL)	0.97 (0.62, 1.90)	0.96 (0.61, 1.79)	1.14 (0.61, 1.95)			
Adiponectin (ng/mL)	4,635 ± 2,545	4,514 ± 2,195	4,353 ± 2,198		0.196	

¹Values are mean ± standard deviation, or median (IQR) if non-normally distributed data. HFCS, high-fructose corn syrup; CRP, C-reactive protein; IL-6, interleukin 6.

²Reflects an overall comparison of the 3 dietary phases by repeated-measures ANOVA.

For our secondary outcome measures, there was no effect of diet on day 9 plasma adiponectin concentrations (Table 3.1). There was no effect of diet on plasma zonulin or LBP (Table 3.2); however, there was a statistically significant effect of diet on the urinary lactulose/mannitol ratio and the percent of lactulose recovered from urine on day 9 ($p < 0.001$ for diet group in overall RM-ANOVA, Table 3.2). Post hoc paired t-tests with Bonferroni adjustment revealed that the lactulose/mannitol ratio following both fructose and glucose diet periods was statistically significantly higher than the ratio following the HFCS diet period ($p < 0.003$), but there was no evidence that the fructose and glucose diet periods differed from each other ($p = 0.228$). The percent of lactulose recovered from urine after the glucose diet period was statistically significantly higher than that recovered after the HFCS diet period. There was also no effect of diet on circulating FFA or fetuin-A (Table 3.2). In sensitivity analyses, exclusion of the six subjects who reported a minor illness (such as a cold) in at least one of the diet periods did not change any of the dietary effects on any of the biomarkers (data not shown).

Table 3.2. Measures of plasma free fatty acids, fetuin-A, and intestinal permeability markers on day 9 of each dietary period¹.

	Fructose (n=24)	HFCS (n=24)	Glucose (n=24)	p (diet)²
Zonulin (ng/mL)	12.78 ± 1.54	12.92 ± 1.49	12.69 ± 1.70	0.366
LBP (ug/mL)	27.7 (19.8, 34.2)	29.8 (21.5, 36.0)	26.1 (21.7, 40.0)	0.387
Lactulose/ Mannitol Ratio	0.047 (0.031, 0.054) ^a	0.031 (0.026, 0.035) ^b	0.043 (0.031, 0.048) ^a	< 0.001
Lactulose recovery (%)	0.25 (0.19, 0.32) ^{a,b}	0.22 (0.19, 0.25) ^a	0.29 (0.24, 0.32) ^b	<0.001³
Free fatty acids (mEq/L)	0.28 (0.20, 0.38)	0.30 (0.17, 0.47)	0.40 (0.24, 0.51)	0.121
Fetuin-A (mg/L)	490 (404, 567)	467 (418, 581)	496 (414, 570)	0.602

¹Values are mean ± standard deviation, or median (IQR) if non-normally distributed data. Values in the same row with different subscript letters are significantly different from each other, $P < 0.05$ (post hoc paired t tests with Bonferroni correction). HFCS, high-fructose corn syrup; LBP, lipopolysaccharide-binding protein.

²Reflects an overall comparison of the 3 dietary phases by repeated-measures ANOVA.

³P-value determined by the non-parametric Friedman test.

Secondary analyses stratified by adiposity showed that overweight/obese subjects had statistically significantly higher plasma CRP ($p=0.029$), IL-6 ($p=0.001$), and LBP ($p=0.005$) compared to normal weight participants (data not shown). However, in analyses conducted to assess whether a statistically significant interaction between adiposity and diet existed, we detected no differential impact of the diets on any of the biomarkers of inflammation in normal weight versus overweight/obese individuals (data not shown).

3.3.2. Adipose tissue inflammation

Fourteen subjects opted to undergo a voluntary abdominal subcutaneous adipose tissue biopsy and their baseline characteristics were not statistically significantly different from that of the

overall study population. Four subjects (1 man, 3 women) were normal weight (BMI of $23.8 \pm 1.0 \text{ kg/m}^2$) aged $36 \pm 15 \text{ y}$, with fasting glucose of $93 \pm 5 \text{ mg/dL}$ and baseline CRP of $0.84 \pm 1.1 \text{ mg/L}$. The other ten subjects (4 men, 6 women) were overweight/obese (BMI of $30.5 \pm 3.4 \text{ kg/m}^2$), aged $41 \pm 13 \text{ y}$, and had fasting glucose of $97 \pm 9 \text{ mg/dL}$, and baseline CRP of $2.7 \pm 1.9 \text{ mg/L}$. Gene expression analyses of samples from these 14 subjects revealed that there was a statistically significant effect of diet on day 9 adipose tissue expression of adiponectin mRNA ($p=0.005$). In *post hoc* t-tests, expression following the fructose diet period was statistically significantly greater than both HFCS ($p=0.048$) and glucose ($p=0.012$) diet periods, after Bonferroni correction for multiple testing. We found no evidence that the adipose tissue adiponectin expression differed following the HFCS vs. the glucose period ($p>1.0$). Nor did we find evidence of an effect of diet on mRNA expression of any of the other genes measured, except for a trend toward a statistically significant difference in CCL2 mRNA expression (Table 3.3).

Table 3.3. Tissue mRNA expression in subcutaneous adipose tissue on day 9 of each diet period in subjects who underwent optional adipose tissue biopsy. Data are in copy number per ng total RNA, normalized to the housekeeping gene GUSB¹.

	Fructose (n=14)	HFCS (n=14)	Glucose (n=14)	p (diet)²
Adiponectin	6,288 ± 1,840 ^a	5,370 ± 1,586 ^b	5,107 ± 1,574 ^b	0.005
TNF-α	1.40 ± 0.58	1.24 ± 0.67	1.44 ± 0.89	0.476
IL-1β	0.41 (0.26, 0.54)	0.32 (0.27, 0.84)	0.33 (0.25, 0.41)	0.596
IL-6	0.53 (0.35, 0.74)	0.51 (0.38, 0.75)	0.49 (0.37, 0.58)	0.492
IL-10	0.66 (0.34, 1.14)	0.95 (0.34, 1.44)	0.90 (0.65, 1.19)	0.149
CCL2	31.5 (22.9, 47.6)	25.3 (19.3, 34.4)	27.2 (22.6, 33.6)	0.056
IFNγ	0.23 (0.15, 0.44)	0.23 (0.17, 0.38)	0.20 (0.15, 0.27)	0.520

¹Values are mean ± standard deviation, or median (IQR) if non-normally distributed data. Values in the same row with different superscript letters are significantly different from each other, $P < 0.05$ (post hoc paired t tests with Bonferroni correction). HFCS, high-fructose corn syrup; GUSB, glucuronidase beta; TNF- α , tumor necrosis factor alpha; IL-1 β , interleukin 1beta; IL-6, interleukin 6; IL-10, interleukin 10; CCL2, chemokine (C-C motif) ligand; IFN γ , interferon gamma.

²Reflects an overall comparison of the 3 dietary phases by repeated-measures ANOVA.

There was no effect of diet on day 9 tissue populations of neutrophils (CD15⁺CD16⁺), total ATMs (CD14⁺CD206⁺), CD11c⁺-ATMs (CD14⁺CD206⁺CD11c⁺), CD4⁺-T-cells (CD3⁺CD4⁺), or CD8⁺-T-cells (CD3⁺CD8⁺) when data were normalized to cells per gram of adipose tissue or as a percent of the CD45⁺ cell fraction (Table 3.4). There was also no effect of diet on the ratio of CD4⁺ to CD8⁺ T cells ($p=0.275$). When cells were normalized as a percent of the CD45⁺ cell fraction, there was a statistically significant effect of diet on the percent of CD1c⁺CD11c⁺ dendritic cells on day 9 ($p=0.017$ for diet group in overall RM-ANOVA, Table 3.4). In *post hoc*

t-tests, dendritic cell numbers following the HFCS diet period was statistically significantly greater than the glucose diet period ($p=0.012$), after Bonferroni correction for multiple testing. We found no evidence that cell numbers differed following the HFCS vs. the fructose period ($p=0.696$) or the glucose vs. the fructose period ($p=0.342$). No unexpected adverse events or adverse events that were more severe than 'mild' occurred in this study.

Table 3.4. Subcutaneous adipose tissue cell populations on day 9 of each diet period in subjects who underwent optional adipose tissue biopsy. For each cell population, data are normalized per gram of tissue and to percent of CD45⁺ leukocytes¹.

	Fructose (n=12)	HFCS (n=12)	Glucose (n=12)	p (diet) ²
CD15⁺CD16⁺ Neutrophils				
10 ³ cells per g SQAT	187 (37, 349)	69 (16, 347)	190 (34, 601)	p = 0.244
as % of CD45 ⁺ fraction	28.5 ± 21.7	18.0 ± 15.9	26.9 ± 20.8	p = 0.254
CD1c⁺CD11c⁺ Dendritic cells				
10 ³ cells per g SQAT	16 ± 6.2	21 ± 15	14 ± 7.3	p = 0.210
as % of CD45 ⁺ fraction	1.95 (1.43, 3.85) ^{a,b}	2.95 (1.93, 4.03) ^a	1.80 (0.90, 2.78) ^b	p = 0.017
CD14⁺CD206⁺ ATM				
10 ³ cells per g SQAT	53 ± 39	62 ± 37	50 ± 35	p = 0.546
as % of CD45 ⁺ fraction	8.33 ± 6.77	9.90 ± 3.92	7.38 ± 4.88	p = 0.320
CD14⁺CD206⁺CD11c⁺ ATM				
10 ³ cells per g SQAT	5.5 (3.3, 13)	14 (2.7, 21)	6.1 (3.2, 12)	p = 0.920
as % of CD45 ⁺ fraction	0.78 (0.56, 1.96)	1.93 (0.57, 3.00)	0.79 (0.31, 1.98)	p = 0.346
	(n=11)	(n=11)	(n=11)	
CD3⁺CD4⁺ T cells				
10 ³ cells per g SQAT	42 ± 14	50 ± 31	57 ± 26	p = 0.401
as % of CD45 ⁺ fraction	6.51 ± 2.83	7.07 ± 3.09	6.91 ± 2.51	p = 0.813
CD3⁺CD8⁺ T cells				
10 ³ cells per g SQAT	39 (25, 54)	30 (20, 106)	45 (30, 58)	p = 0.580
as % of CD45 ⁺ fraction	6.75 ± 4.18	6.82 ± 3.81	6.52 ± 4.20	p = 0.949
CD4⁺/CD8⁺ T cell ratio	0.93 (0.61, 1.31)	1.01 (0.79, 1.39)	1.07 (0.72, 1.47)	p = 0.275

¹Values are mean ± standard deviation, or median (IQR) if non-normally distributed data. Values in the same row with different superscript letters are significantly different from each other, $P < 0.05$ (post hoc paired t tests with Bonferroni correction). HFCS, high-fructose corn syrup; ATM, adipose tissue macrophage; SQAT, subcutaneous adipose tissue.

²Reflects an overall comparison of the 3 dietary phases by repeated measures ANOVA.

3.4. Discussion

The major finding from this study was that we found no evidence that 8-d consumption of fructose- vs. HFCS- vs. glucose-sweetened beverages differentially affected our primary outcome, fasting plasma CRP and IL-6 as measures of systemic inflammation, in normal weight to obese adults. Acutely, the substantial overconsumption of energy in individuals consuming these SSBs did not trigger an increase in systemic inflammation. We also did not detect any difference among diet periods in measures of adipose tissue inflammation. Together, our data suggest that neither the short- to medium-term consumption of excess energy nor excessive amounts of fructose *per se* trigger low-grade, chronic inflammation.

Our study was motivated by strong evidence from rodent models in which high fructose but not glucose consumption led to increased intestinal permeability and translocation of bacterial lipopolysaccharide (LPS) into the portal vein. Elevated portal vein LPS triggered inflammation in the liver via a mechanism that was dependent on the LPS-receptor (toll-like receptor 4) as well as the presence of bacteria in the gut (98). Furthermore, treatment of fructose-fed rodents with antibiotics reduced lipid accumulation and inflammation in the liver suggesting that both may result directly from exposure to intestinally-derived endotoxin (99).

Contrary to these findings, and consistent with a lack of evidence of a differential effect on biomarkers of inflammation, we saw no difference in the plasma markers of endotoxin exposure (LBP) or gut tight-junction integrity (zonulin) following the three diet periods. We also found no clear evidence that fructose as compared to glucose increases intestinal permeability, as measured by the lactulose-mannitol test. A higher urinary L/M-ratio suggests that lactulose, a large diameter molecule that is normally not absorbed in the healthy human gut, has passed paracellularly, or unregulated, into the portal circulation. Elevation of this ratio is indicative of increased intestinal permeability and suggests that other luminal contents, such as bacteria-derived molecules, could pass unregulated into the portal circulation as well. While the reduced L/M-ratio at the end of the HFCS-phase may seem notable, several considerations lead us to reject our hypothesis that fructose has specific effects on intestinal permeability that differ from those of glucose. First, we found no evidence that either the L/M-ratio or the lactulose recovery differed between the fructose and glucose periods. Second, we detected not even the slightest trend towards differences in the other measures of intestinal permeability (plasma zonulin and

LBP). And third, albeit statistically significantly different, the L/M-ratio was within the range that is considered normal, and well below that observed in clinical conditions associated with intestinal permeability such as celiac disease (128). And fourth, for any difference in intestinal permeability to be clinically relevant (at least in the context of our hypothesis), differences would need to be associated with differences in measures of systemic inflammation, which was not the case here.

Few intervention studies have been carried out in humans investigating SSBs and systemic inflammation and the results have been discrepant. In line with our findings, Cox et al. (103) observed no change in plasma CRP or IL-6 in subjects consuming fructose versus glucose beverages at 25% of calories for 10 weeks. However, MCP-1, PAI-1, and E-selectin, also pro-inflammatory mediators, all increased to a statistically significant degree from baseline following fructose- but not glucose-beverage consumption (103). Total body weight and subcutaneous adipose tissue increased slightly but similarly in both groups, but visceral adipose tissue increased in the fructose-beverage group only (81). Visceral, rather than subcutaneous, adipose tissue is the primary determinant of circulating MCP-1 and PAI-1 concentrations (104, 105). Hence, it is possible that the expansion of visceral adipose tissue in individuals drinking fructose-sweetened beverages in that study was the primary driver of an increase in these specific markers of inflammation, rather than a direct effect of fructose. Further supporting this hypothesis, Silbernagel et al. (102) conducted a 4-week parallel design study comparing high-glucose versus high-fructose consumption on plasma PAI-1, MCP-1, E-selectin, and CRP in healthy adults. Subjects consumed 150 g/d of either glucose or fructose dissolved in water, a dose similar to the one used in our study. After 4 weeks, there were no statistically significant changes in plasma PAI-1, MCP-1, E-selectin or CRP after either intervention, nor were any differences detected between treatments (102). Notably, subjects in both the fructose and glucose groups gained a similarly small amount of weight, yet visceral adipose tissue mass did not change (102). In a randomized, cross-over study, Aeberli and colleagues (39) tested whether 80 g versus 40 g per day of glucose, fructose, or sucrose differentially influenced plasma CRP compared to dietary advice to consume a low-fructose diet. Compared to pre-intervention baseline levels, fasting CRP was statistically significantly increased after all intervention phases including the low-fructose diet control phase (39). While it remains unclear why CRP

concentrations increased in all study periods, these data are consistent with our finding of no evidence of a differential effect of fructose vs. glucose on systemic inflammation.

We also investigated a novel mechanism of adipose tissue inflammation involving the FFA chaperone protein fetuin-A (91). We hypothesized that adipose tissue inflammatory pathways could be triggered directly by fructose-induced systemic inflammation (118), or indirectly by fetuin-A activation of inflammatory pathways in adipose tissue (120). We measured several genes in adipose tissue known to be involved in the inflammatory process, and quantified and characterized the activation state of immune cell populations isolated from the SVC fraction, in a comprehensive effort to detect any differential effect of the beverages on adipose tissue inflammation. We saw no evidence of a consistent difference in the markers of adipose tissue inflammation that would suggest that fructose has specific pro-inflammatory effects on adipose tissue. While we detected statistically significantly lower numbers of dendritic cells in adipose tissue after subjects had consumed the glucose- as compared to the HFCS-sweetened beverages, our data showed no evidence of a difference between glucose- and fructose-periods, and no consistent changes in any other measure of adipose tissue inflammation. One finding that may be notable was that adiponectin gene expression was statistically significantly greater following the fructose diet period compared to both the HFCS and glucose diet periods. Adiponectin is an insulin-sensitizing, anti-inflammatory adipokine that is inversely correlated with adiposity. It seems possible that overexposure of adipocytes to glucose may directly or indirectly lead to a suppression of adiponectin gene expression. The clinical relevance of this finding is unclear, however, because this change in subcutaneous adipose tissue expression of adiponectin was not associated with a similar change in plasma adiponectin concentrations. This may have been due to the fact that plasma adiponectin is preferentially expressed in intra-abdominal adipose tissue, which may not have been differentially affected by the three diets.

The crossover design was a major strength of our study because the influence of the substantial inter-individual differences in biomarkers of inflammation and gene expression was minimized by this approach. Furthermore, this is the first study to our knowledge to screen for and enroll only those subjects with normal fructose absorption. The relatively short duration of the intervention was potentially both a strength and limitation of the study. Because subjects did not gain weight, we were able to measure the effects of the beverages on inflammation in a body

weight-independent fashion. However, it is also possible that the markers we chose to study do not change to a statistically significant degree within this relatively short time period. It is also important to emphasize that our study design is strongest to compare the data generated at the end of each diet period, and less so to assess changes from baseline during each dietary period.

In conclusion, our data show no evidence of a differential effect of excessive amounts of fructose vs. HFCS vs. glucose from SSBs consumed over 8 days on low-grade chronic systemic inflammation in normal weight to obese adults.

4. SUMMARY

Sugar-sweetened beverage consumption is strongly associated with both obesity and cardiometabolic disease. As the association between SSB intake and cardiometabolic disease is at least partly mediated by adiposity, many studies in this field have attempted to identify whether a body weight independent association also exists such as through a unique effect of fructose on risk factors for cardiometabolic disease. Previous studies in this area have all had limitations that have prevented these questions from being answered definitively; therefore the overall aim of this project was to determine whether fructose, as compared to glucose, consumed in the form of an SSB, uniquely influences energy homeostasis and/or systemic inflammation and might be a primary driver of the observed associations between SSB intake and cardiometabolic disease. We also investigated whether body-weight independent effects exist that explain the associations, or if the relationship might be mediated solely by an increase in adiposity. We addressed weaknesses in previous studies by: 1) implementing a double-blind crossover design, 2) providing all foods to subjects for the entirety of the intervention yet still allowing subjects to be ‘free living’, 3) including glucose-, fructose-, HFCS-, and aspartame-sweetened test beverages, 4) utilizing a more robust screening procedure to exclude those individuals with fructose malabsorption, and 5) incorporating short-to-medium term intervention periods in order to minimize weight gain. As such, our design allowed us to disentangle the impact of glucose from that of fructose on energy homeostasis and biomarkers of chronic disease, while also helping us shed light on the question of whether SSBs are associated with increased weight gain because of their liquid calorie nature or their high fructose content.

4.1. Fructose-, glucose-, and HFCS-sweetened beverage consumption promotes an increase in energy intake compared to aspartame

The major finding from the pilot study (n=10) was that healthy normal-weight adults consumed a statistically significantly greater number of calories when 25% of their estimated energy requirement was provided as a glucose- or fructose-sweetened beverage compared with an isovolumetric amount of an aspartame-sweetened beverage. On average, caloric intake occurred approximately 20% and 17% in excess of energy needs when subjects consumed fructose vs. glucose beverages, respectively, suggesting that the participants compensated only for about one-third of the calories consumed in the glucose- and fructose-sweetened beverages.

In the larger follow-up study (DASI, n=24), we hypothesized that fructose-sweetened beverages would promote a greater increase in energy intake compared to HFCS- or glucose-sweetened beverages when normal weight to obese adults consumed 25% of their estimated energy requirement in the form of an SSB in addition to an *ad libitum* diet. Given that fructose does not stimulate insulin secretion, we speculated that the downstream hormonal responses controlling energy homeostasis would also be blunted when pure fructose, or fructose-containing beverages, were consumed. While evidence suggests this is the case over the short term (24-hrs) (76), we found that this does not translate into measurable differences in energy intake when fructose- as compared to glucose-sweetened beverages are consumed over the medium term (8-d) (121). We did observe an increase in overall energy intake during all diet periods, however our data show no evidence of a difference among the diets. In fact, participants consistently consumed 16% greater energy on average than their estimated energy requirements predicted they would need to maintain energy homeostasis while consuming the beverages. We concluded that the positive energy balance we observed over the short term would be expected to lead to weight gain and adiposity over time.

We would not expect, however, that individuals would maintain the same degree of caloric excess day after day for the duration of time that SSBs are consumed. Clearly, this would result in adiposity far in excess of what is observed in those who habitually consume SSBs over the long term. In healthy individuals, body weight and energy homeostatic compensatory mechanisms are triggered in response to an increase in energy intake exceeding that which is required to maintain a body adiposity 'set point'. While the degree to which body weight can be maintained in the context of excess energy intake varies widely among individuals, the activity of several energy expenditure pathways is increased in response to overfeeding. For example, non-exercise physical activity and other energy wasting pathways, such as uncoupling proteins in brown adipose tissue and skeletal muscle or activation of futile substrate cycles that consume ATP, have been shown to increase in the context of excessive energy intake (129). We speculate that this would also be the case in response to long-term SSB intake, although perhaps the energy homeostasis compensatory mechanisms are lagged or mismatched to a small degree, resulting in slow, gradual increase in adiposity. Evidence suggests that this could indeed be the case, given that a recent meta-analysis concluded that a 1 serving per day increase in SSB intake was associated with an additional average weight gain of 0.12 kg per year (12), rather than several

kilograms that would be expected if these individuals consistently consumed 16% greater energy above their energy requirements. We also observed evidence of energy homeostatic mechanisms attempting to control energy intake in the DASI study. We tested whether there were any differences in calorie intake between the first and second 4-day diet blocks within each 8-day period. We found that overall energy intake was statistically significantly greater by 87 ± 507 kcal during the first 4 days of the diet periods compared to during the second 4 days ($p = 0.003$ for the difference between the first and second 4-day block in repeated measures ANOVA), with no evidence of a difference in this reduction in energy intake among the three dietary periods (121).

Overall, we concluded that the findings from both studies support the liquid calories hypothesis and suggest that it is sugars consumed in liquid form, rather than the relative amounts of fructose vs. glucose in the beverages, that promote an overall increase in energy intake by failing to invoke a concomitant reduction in energy intake from solid foods.

4.2. No evidence that beverages sweetened with fructose, glucose, or HFCS differentially affect chronic, low-grade inflammation

4.2.1. No evidence of a differential effect of fructose- vs. HFCS- vs. glucose-sweetened beverages on systemic inflammation

The primary aim of the DASI study was to determine if 8-d consumption of beverages sweetened with fructose vs. HFCS vs. glucose at 25% of estimated energy requirements differentially impact biomarkers of systemic inflammation (fasting plasma CRP and IL-6) in normal weight to obese adults. We hypothesized that fructose would uniquely trigger an increase in inflammation compared to glucose beverages (over an 8-d period) and that there would be a differential effect of the beverages on inflammation (at day 9 of each period). This hypothesis was based on evidence from the pilot study ($n=10$), in which fasting plasma CRP and IL-6 concentrations were 0.92 ± 0.64 mg/L and 0.60 ± 0.40 pg/mL, respectively, higher after subjects had consumed the fructose-sweetened beverage compared to the glucose-sweetened beverage (Kuzma, et al. submitted). However, in the larger follow-up study (DASI, $n=24$), despite the overconsumption of energy, we saw no evidence that the SSBs triggered an increase in systemic inflammation nor did we detect any difference in biomarkers of inflammation among diet periods. We concluded that neither the short- to medium-term intake of excess energy nor excessive fructose intake *per se* triggered an increase in systemic inflammation, and that our data provide no evidence that

fructose differentially affects measure of systemic inflammation in the short to medium term compared to glucose.

4.2.2. No evidence that fructose-sweetened beverages promote intestinal permeability compared to glucose-sweetened beverages

A secondary aim of the DASI study was to determine whether 8-d consumption of beverages sweetened with fructose vs. HFCS vs. glucose at 25% of estimated energy requirements would promote an increase in intestinal permeability. We hypothesized that biomarkers of intestinal permeability (urinary lactulose/mannitol ratio and plasma zonulin) and a biomarker of bacterial LPS exposure would be highest following the fructose diet period compared to the HFCS and glucose periods. Given the strong data in rodent models suggesting that fructose promotes systemic inflammation through an increase in intestinal permeability and the translocation of bacterial contents from the gut lumen into systemic circulation (98, 99), we hypothesized that this novel mechanism might operate in a similar manner in humans.

Contrary to these prior findings in rats, but consistent with our null results in regard to systemic inflammation, we found no evidence of a difference in biomarkers of gut tight-junction integrity or plasma markers of endotoxin exposure following the three diet periods. We did, however, find that the lactulose/mannitol ratio was statistically significantly lower following the HFCS diet period compared to both the glucose and fructose phases. Yet despite this finding, we concluded that fructose does not trigger an increase in intestinal permeability differently than glucose. Inherent in our study design, we outlined *a priori* that measures of intestinal permeability and systemic inflammation endpoints were to be interpreted together and that in order to detect an increase in intestinal permeability, we would also observe statistically significant trends in several endpoints. Furthermore, as our hypothesis predicted a fructose effect, we would have expected to see the lactulose/mannitol ratio following the HFCS phase to be intermediate to that measured after the glucose and fructose phases, which was clearly not the case. For these reasons, and because the lactulose/mannitol ratio measured after the fructose and glucose phases did not exceed the range that is considered clinically normal, we concluded that our data provide no evidence that consumption of fructose-sweetened beverages differentially increases intestinal permeability compared to glucose-sweetened beverages.

4.2.3. No evidence that fructose- vs. HFCS- vs. glucose-sweetened beverages differentially affect adipose tissue inflammation

A secondary aim of the DASI study was to determine whether 8-d consumption of beverages sweetened with fructose vs. HFCS vs. glucose at 25% of estimated energy requirements would differentially impact adipose tissue inflammation in normal weight to obese adults. We hypothesized that both adipose tissue expression of genes involved in the inflammatory process, as well as the adipose tissue content of immune cell populations, would be elevated in response to increasing fructose content of the beverages. We expected to observe the highest level of pro-inflammatory gene expression and cell populations following the fructose-beverage phase compared to the glucose phase, with the HFCS phase intermediate between the two. This hypothesis was based on evidence that adipose tissue inflammation can be triggered by body weight gain (56) as well as by systemic inflammation (118), and potentially increased intestinal permeability and endotoxemia. We also hypothesized that fructose might promote adipose tissue inflammation through a novel mechanism. Based on evidence supporting a positive association between fructose intake and hepatic de novo lipogenesis (81), we speculated that fructose might also induce adipose tissue inflammation by influencing the synthesis of the hepatokine fetuin-A.

With the exception of a statistically significant increase in the percentage of CD11c⁺CD11b⁺ dendritic cells within the leukocyte fraction following the HFCS phase compared to both the fructose and glucose phases, we saw no evidence of a differential effect of fructose consumption on adipose tissue inflammation. We observed a statistically significant difference in the insulin sensitizing adipokine adiponectin, which appeared to be suppressed according to the glucose content of the beverages. However, when considering all the endpoints together, we saw no evidence of a consistent difference in markers of adipose tissue inflammation that would suggest that fructose has a particularly pro-inflammatory effect on adipose tissue. Furthermore, our data provide no evidence of a difference in the plasma concentration of fetuin-A among the diet periods, which further supports our overall null findings in regard to the effect of fructose- vs. HFCS- vs. glucose-sweetened beverages on adipose tissue inflammation.

4.3. Conclusion and recommendations

Considering the results of both the pilot and DASI studies together, in the context of the well-controlled randomized, cross-over design, with the provision of all foods for the duration of the study and the exclusion of individuals with fructose malabsorption, we can be quite confident in

our conclusion that 1) SSBs promote an increase in overall energy intake in normal weight to obese adults over the medium term; that 2) our data provide no evidence that fructose and glucose differ in terms of their ability to regulate energy intake when consumed in the form of an SSB; 3) rather, the liquid nature of these sugars promotes excess energy intake by failing to invoke a concomitant reduction in energy from solid foods; 4) despite excessive overall energy intake, we saw no evidence of an increase in low-grade inflammation over 8 days; and 5) our data do not show that excessive short-term fructose consumption differentially elevates systemic inflammation, adipose tissue inflammation, or intestinal permeability compared to HFCS or glucose when consumed in the form of an SSB.

In light of these findings, we believe there is sufficient evidence to implicate SSBs as a causal factor in body weight gain through the promotion of excess energy intake. However, we did not find that the consumption of fructose beverages, despite its unique properties, promotes deleterious health consequences differently than glucose beverages. In general, our findings are in line with the conclusions presented by Sievenpiper et al. (87) in their meta-analysis of controlled feeding trials investigating the effect of fructose on body weight. Their analysis included 31 trials in which fructose was substituted for other non-fructose carbohydrate in an isocaloric manner, and 10 trials in which fructose was added to a control or habitual diet such that fructose provided excess energy relative to the diet alone (hypercaloric). In the hypercaloric trials, fructose was provided in doses of 104 to 250 g/d, or 18% to 97% of daily energy intake. Not surprisingly, the isocaloric trials provided no consistent evidence of a body weight-increasing effect of fructose while the hypercaloric trials resulted in statistically significant weight gain of 0.53 kg on average over a median follow-up of 1.5 weeks (87). In the context of these hypercaloric trials, it is difficult to determine whether the relative contribution of fructose, excess energy, or a combination of both might be contributing to body weight gain. However, it appears that the weight gain that did occur was similar to that which would be expected from a diet supplemented with the same amount of excess energy supplied in any form. Therefore, the overall conclusion was that the effect of fructose on body weight is not different from that of other carbohydrates when diets providing equal amounts of energy are compared (87).

Overall, our findings support the conclusions of the meta-analysis and we believe the link between SSB intake and chronic disease is most likely a result of excess energy intake due to

consumption of sugars in liquid form rather than a specific body weight-independent effect of fructose operating through pro-inflammatory pathways. We would recommend that the consumption of sugars in liquid form be minimized in order to best maintain energy homeostasis and avoid increased risk of cardiometabolic disease.

4.4. REFERENCES

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