

Effects of Differentially Sweetened Beverages on Hepatic and Adipose De Novo Lipogenesis in
Healthy Young Adults

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Abstract

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Added sugars from sweetened beverages contribute a substantial number of calories to the American diet with numerous associated negative health effects. There is a need to evaluate the different effects of fructose, glucose, and aspartame on healthy young adult metabolism to better understand the etiology of obesity and obesity-associated diseases. This study aims to evaluate differences in *de novo* lipogenesis in adipose tissue versus the liver when subjects consume differentially sweetened beverages, based on research in rodent models suggesting that the balance of hepatic vs. adipose tissue lipogenesis has substantial effects on energy and glucose homeostasis. Healthy young adults consumed a controlled and standardized diet with glucose-, fructose-, or aspartame-sweetened beverages as 25% of caloric intake for 8 day periods in a randomized double-blind cross-over study. After each diet period, subjects were admitted to the University of Washington Clinical Research Center where fasting plasma samples were obtained for later gas chromatographic measurement of palmitoleic acid in both phospholipid and free fatty acid fractions as a biomarker for *de novo* lipogenesis, in addition to lipid levels and inflammatory markers. There was no significant difference in

palmitoleic acid levels in the free fatty acid fraction of plasma between the three diet periods ($p=0.740$). There was a significant difference in palmitoleic acid levels in the phospholipid fraction of plasma between the three diet periods ($p=0.020$). In *post hoc* analysis, there was significantly higher palmitoleic acid level in phospholipids in the fructose- than in the aspartame-sweetened beverage diet period ($p=0.024$, adjusted). These results indicate that while there was no significant difference in the effect of the different sugars on adipose tissue *de novo* lipogenesis, the sugars elicited differential effects on hepatic *de novo* lipogenesis. Phospholipid palmitoleic acid was positively associated with plasma fasting triglycerides, and inversely with resting energy expenditure. Taken together, these results suggest that dietary fructose, but not glucose, induces hepatic *de novo* lipogenesis and has detrimental effects for metabolic health.

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Chapter 1: Literature Review

Introduction

Obesity is an increasing public health problem due to the number of obesity-associated diseases such as fatty liver disease, type 2 diabetes, cardiovascular disease, and some types of cancer. Obesity is defined as a body mass index (BMI) in adults greater than 30 kg/m². In 2007-2008, 32.2% of adult men and 35.5% of adult women in the United States were obese (2). By 2030 it is estimated that 65 million additional obese adults in the United States will incur an estimated additional 6-8.5 million cases of diabetes, 5.7-7.3 million cases of heart disease and stroke, and 492,000-669,000 cancer cases, at an additional medical cost of \$48 to \$66 billion per year (3). The economic, health, and social burden of obesity mandates that we better understand factors contributing to the development of obesity, as well as the etiology of obesity-associated chronic diseases.

Changes in the contemporary dietary pattern have likely contributed to a rise in obesity. While it is natural for food supply and consumption to change as technology, climate, and populations change, recent shifts in dietary patterns have been rapid and dramatic. In 2009, Americans ate significantly more grain, fruit, caloric sweeteners, cheese, and added fats, while eating less red meat, eggs, and milk compared to 1970 (4). In addition to consuming more of these food groups, food is increasingly processed and packaged, with longer and less familiar ingredient lists, many of which may have unknown health consequences. Increased fructose consumption through high-fructose corn syrup (HFCS) or sugar is one modern trend of potential

relevance for obesity and associated disease. As we will see below, the increased consumption of fructose may in and of itself be associated with obesity. Fructose may also have further implications to chronic disease risks independent of obesity (5). Fructose is naturally found in fruits and honey and was only introduced as a sweetener in the mid-1800s. While fructose is found in regular table sugar, consumption of fructose remained fairly low until the advent of HFCS. With the introduction of HFCS in the late 1970s, soda consumption and fructose-sweetened foods dramatically increased and since then fructose figures prominently as a caloric sweetener (5). In general, Americans consume the majority of fructose in the form of added sugars, including fructose-sweetened beverages; one 12 ounce soda may have 22 g of fructose (40 g HFCS) and this only accounts for partial daily fructose consumption in the modern world (6). Fructose found in the form of added sugars, either as HFCS or sucrose, is present in high levels rivaling any intrinsic levels in whole foods and, on average, contribute 16% of daily calories for Americans, primarily in the form of sweetened beverages, which correspond to 90 grams of added sugars daily (7). Fructose prevails in the American diet in foods ranging from soda, bread, desserts, and ketchup, often in the form of HFCS.

With the introduction of agriculture, animal domestication, and later industrialization, the food supply has shifted from few or no added sugars to highly processed foods with abundant added sugar, in addition to a number of other changes (8). In England, annual per capita consumption of fructose from refined sucrose was 3.4 kg in 1815, likely with a similar level in the United States (8). Fructose consumption from both HFCS and from sucrose was 23.1 kg per person in 1970 and 28.9 kg in 2000 (8). Such a shift in a relatively short time has

been suggested to contribute to the increase in a number of common chronic diseases, including those associated with obesity (8). The increase in obesity rates has mirrored the increase in sugar and sugar-sweetened beverages since the 1970s.

Epidemiologic evidence ties added sugar intake to obesity, and in turn, type 2 diabetes, cardiovascular disease, and metabolic syndrome. In an analysis of the Nurses' Health Study, sugar-sweetened beverages were related to an increase in body weight, second to potato chips and potato products (9). Long-term consumption of sugar-sweetened beverages could indicate overall less healthy patterns, but also seems to independently lead to weight gain (9). Women with stable consumption typically did not have a change in weight gain, but those who increased sugar-sweetened beverage consumption over a four year period had greater weight gain (10). Additionally, women who had one or more beverage a day had a relative risk of type 2 diabetes of 1.83 compared to less than 1 beverage per month (10). Coronary heart disease has also been associated with regular consumption of sugar-sweetened beverages (11). The mechanism is partly related to excess calories and increased adiposity, but also fructose could independently play a role through the unique way in which it is metabolized. Fructose may be a major piece of the puzzle contributing to the array of afflictions known as metabolic syndrome.

This assertion is supported by numerous recent findings. Fructose has been implicated in a number of health effects, including increased triglycerides, insulin resistance and fatty liver (12-14). Fructose is absorbed through GLUT5 or GLUT2 transporters and directly metabolized by the liver (5). Fructose bypasses regulatory checkpoints in the glucose metabolic pathway,

and does not induce an insulin response (5). These and other acute effects of fructose, and differences to glucose, will be discussed in the next section, before we move to a discussion of the effects of longer-term consumption of diets rich in fructose in the following section.

Acute effects of dietary fructose

There is much research on differing diet compositions of varied fat, carbohydrate, and protein content, but the acute effects of different carbohydrates are just beginning to be studied extensively. In a 2004 study, normal-weight women were fed a diet of 55, 30, and 15% of calories as carbohydrate, fat, and protein, respectively, with 30% of calories as fructose- or glucose-sweetened beverages (1). High fructose consumption resulted in lower plasma glucose and insulin levels throughout the day, as seen in Figures 1.1 and 1.2 (1). Fructose is removed from the circulation by the liver, with peripheral blood fructose levels remaining low and largely unchanged following a meal high in fructose (1). In the liver, fructose induces *de novo* lipogenesis, which increases triglycerides, lowers HDL levels, and leads to decreased localized and systemic insulin sensitivity (1, 15, 16). Glucose-sweetened beverages induced a drop in triglycerides after postprandial peaks and during overnight fasting (1). In addition to changes in insulin and *de novo* lipogenesis, acute fructose consumption compared to glucose consumption induces adverse changes in satiety factors.

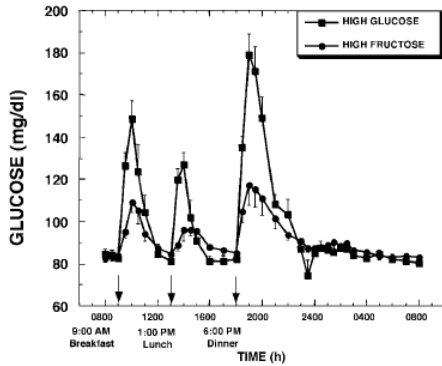


Figure 1.1 Plasma glucose concentrations on diets rich in fructose or glucose (1).

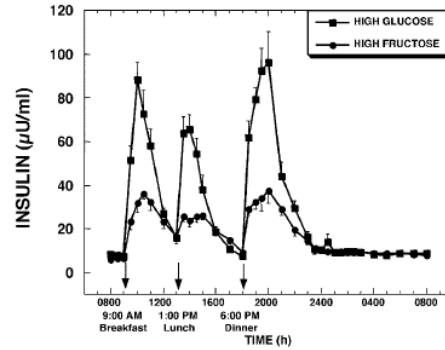


Figure 1.2 Plasma insulin concentrations on diets rich in fructose or glucose (1).

Leptin, which plays a crucial role in long-term regulation of calorie intake and energy homeostasis, increases more in quantity and rate after consumption of glucose than fructose, as shown in Figure 1.3 (1). Insulin-mediated glucose uptake into fat cells stimulates leptin synthesis and secretion; thus, lower levels of insulin and glucose in a high-fructose diet are associated with lower leptin levels throughout the day (1). Chronically low leptin levels are shown to decrease energy expenditure and often results in excess calorie consumption, which illustrates the likely benefit of higher leptin levels following glucose consumption (17). The authors predicted that plasma ghrelin levels would be greater after high fructose consumption due to reduced insulin-mediated suppression of ghrelin post-prandially. As shown in Figure 1.4, plasma ghrelin levels were indeed generally higher after fructose consumption, but this trend was not statistically significant, likely due to large degrees of within-person variation (1).

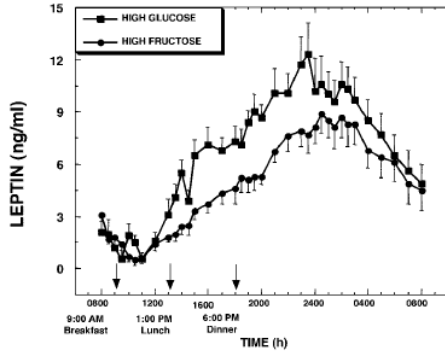


Figure 1.3 Plasma leptin concentrations on diets rich in fructose or glucose (1).

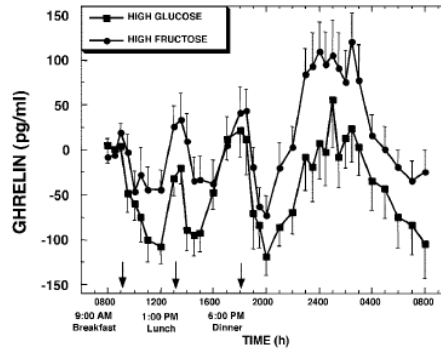


Figure 1.4 Plasma ghrelin concentrations on diets rich in fructose or glucose (1).

The short term effects of high fructose consumption indicate that calories from fructose do not stimulate the same physiologic response as calories from glucose. This has short term effects on the hormonal levels regulating energy homeostasis, with the potential for long term implications. In combination, these physiological responses in the short term could lead to muted satiety cues and greater caloric intake. Because fructose does not stimulate insulin secretion, it is possible that higher calorie intake and ultimate weight gain could be linked to lower insulin and leptin and higher ghrelin levels in fructose-fed individuals (1).

Long term effects of dietary fructose

In addition to the short-term effects of fructose consumption, longer-term consumption of fructose-sweetened beverages has the potential to adversely affect hormones involved in energy homeostasis. After ten weeks of consuming 25% of calorie requirements as fructose- or glucose-sweetened beverages, and otherwise consuming the study diet or their habitual diet *ad*

libitum, older overweight men and women exhibited similar responses to acute fructose consumption. The mean glucose and insulin diurnal amplitudes were lower in response to drinking fructose-sweetened beverages even after subjects had consumed such beverages for as much as 10 weeks (18). While short-term fructose consumption resulted in lower plasma glucose levels, in this 10-week study, fasting glucose and insulin concentrations were higher at the end of the diet phase when subjects had consumed fructose-sweetened beverages compared to glucose-sweetened beverages (18). The higher fasting glucose and insulin levels at the end of the study suggest a reduction in insulin sensitivity and glucose tolerance. Insulin sensitivity was decreased after subjects had completed the 10-week diet period that included the consumption of 25% of total calories in the form of a fructose-sweetened beverage (18). In addition, subjects had lower postprandial lipoprotein lipase (LPL) activity, indicating a delay in triglyceride clearance, partially explaining higher triglyceride levels after long-term fructose consumption (18). After 10 weeks of consumption of 25% of calorie intake as fructose or glucose-sweetened beverages, overweight and obese subjects had higher triglycerides, higher fasting LDL and apoB concentrations (12). In addition to differences in the change in blood profiles of subjects consuming diets high in fructose or glucose, subjects also had changes in *de novo* lipogenesis in the liver.

In a study evaluating changes in obesity of human subjects consuming either glucose or fructose, central adiposity increased in subjects fed 25% of daily calories as fructose for 10 weeks and hepatic *de novo* lipogenesis increased with fructose consumption (12). An increase in hepatic *de novo* lipogenesis leads to higher triglyceride formation with fructose consumption

(12). In this study, only postprandial hepatic *de novo* lipogenesis was higher, while fasting hepatic *de novo* lipogenesis was not affected (12). Fructose, compared to glucose, also induces an increase in intra-abdominal fat mass (12). Glucose, when consumed in excess, appears to increase subcutaneous fat, but not visceral fat (19). A change in fat deposition and lipogenesis from different carbohydrates influences components of metabolic syndrome. These authors demonstrated that chronic consumption of fructose-sweetened beverages, but not glucose-sweetened beverages, trigger the metabolic syndrome in healthy subjects.

Fructose's metabolic shunt bypassing peripheral blood likely on diets rich in fructose versus glucose means that hepatic or adipocyte *de novo* lipogenesis are affected differently. This is a possible mechanism to explain changes in triglyceride levels linked to fructose consumption (1). As we will see in the next section, hepatic as compared to adipose tissue *de novo* lipogenesis may have varied consequences for glucose and energy homeostasis.

Effects of hepatic versus adipose tissue *de novo* lipogenesis on glucose and energy homeostasis

If, as the findings summarized above suggest, glucose and fructose differentially affect *de novo* lipogenesis in adipose tissue vs. the liver, it may be of interest to ask the question whether this has implications for energy homeostasis or metabolic health. Two recent papers provide some important insight in this regard. The group of Gökhan Hotamisligil evaluated *de novo* lipogenesis in mice that were double knockouts for fatty acid binding proteins 4 and 5 (FABP4, 5, or aP2 and mal1) (20, 21). Fatty acid binding proteins (FABP) are lipid chaperones,

with FABP4 and 5 being the major forms present in adipocytes (20, 21). One may hypothesize that a lack of the major lipid transporters in adipocytes would impair lipid storage in fat tissue, leading to substantial ectopic fat deposition associated with metabolic disease. Interestingly, however, mice lacking both FABP4 and 5 (FABP^{-/-} KO) were resistant to diet-induced obesity, despite eating a greater number of calories, and had improved insulin sensitivity and less fat accumulation in the liver (21).

In evaluating a mechanism for these unexpected results, investigators noted that FABP^{-/-} KO mice had higher concentrations of cis-palmitoleic acid (C16:1) in adipose tissue and the free fatty acid fraction of plasma (21). Cis-palmitoleic acid is a product of *de novo* lipogenesis, and its presence in adipose tissue and free fatty acid indicates an increase in adipose tissue *de novo* lipogenesis. Palmitoleic acid is synthesized in adipose tissue or the liver and is only found in very low levels in the diet, making it a good biomarker for *de novo* lipogenesis (20). In FABP^{-/-} KO mice, higher levels of palmitoleic acid and free fatty acids were associated with improved insulin sensitivity and greater energy expenditure, suggesting a possible regulatory role of palmitoleic acid in energy and glucose homeostasis (20). Considering that palmitoleic acid is an adequate biomarker of *de novo* lipogenesis, it could be used to assess dietary influences on *de novo* lipogenesis.

An increase in adipocyte *de novo* lipogenesis was hypothesized to be up-regulated in the FABP^{-/-} KO mice because influx and sensing of long-chain fatty acids was diminished due to lack of the chaperone molecules FABP4 and FABP5. The lack of signaling for blocking lipogenesis

resulted in an increase in lipogenesis, with the unexpected result of an improvement in metabolic health. As a potential explanation, these authors found that palmitoleic acid inhibited hepatic *de novo* lipogenesis and triggered β -oxidation of fatty acids in the liver, while adipose *de novo* lipogenesis increased (21). Increased β -oxidation in the liver offers an explanation for decreased triglycerides in the liver, and potentially for increased energy expenditure and improved insulin sensitivity.

FABP4, 5^{-/-} KO mice had greater plasma levels of palmitoleic acid and were resistant to diet-induced obesity, on either high fat or regular diets. The double knockout mice were leaner than wt mice, but ate slightly more food on regular or high-fat diets (21). Rectal temperatures and fecal lipid content did not differ, suggesting that energy expenditure must be greater in knockout mice through another mechanism, either through an increase in metabolic processes, postprandial thermogenesis, or physical activity (21). Resting energy expenditure was higher in FABP^{-/-} KO mice, explaining how the KO mice ate more calories but gained less weight. The differences between knockout and wt mice suggest that the balance of *de novo* lipogenesis in the liver versus adipose tissue is highly relevant for the regulation of energy and glucose homeostasis.

The results from these experiments in mice suggest a benefit from adipose tissue *de novo* lipogenesis and it is therefore of interest to investigate influences on changing the balance of hepatic and adipose tissue *de novo* lipogenesis on humans. *De novo* lipogenesis in human adipose tissue occurs at lower levels than in rodents, but is still active and contributes to the

body pool of fatty acids (22). Interestingly, lean individuals have higher levels of adipose tissue *de novo* lipogenesis than obese individuals, who have higher levels of hepatic *de novo* lipogenesis (23, 24). It is interesting to evaluate the role of different dietary factors in changing the balance in liver versus adipose *de novo* lipogenesis in humans. One such factor may be different dietary sugars. It has been demonstrated that fructose but not glucose has a number of adverse metabolic effects, yet the mechanism is not fully understood. Following fructose consumption, fructose levels remain relatively low in the peripheral blood due to immediate processing by the liver. In contrast, glucose consumption induces a rise in blood glucose levels, followed by an increase in insulin. Fructose is processed immediately in the liver and increases hepatic *de novo* lipogenesis, while glucose may be a metabolite for adipocyte *de novo* lipogenesis. Glucose consumption, compared to fructose consumption, results in increased beta-oxidation of fats in the liver and higher plasma glucose levels, improving insulin sensitivity, compared to glucose consumption (5). Taken together, these observations may be explained by a differential effect of fructose vs. glucose on *de novo* lipogenesis in the liver (fructose) vs. adipose tissue (glucose). Palmitoleic acid may be used as an indicator of differential *de novo* lipogenesis and offer some explanation to glucose and fructose's effects on the body.

It is possible that palmitoleic acid acts as a lipokine to increase energy expenditure and hepatic fat oxidation, decrease hepatic fat content, and improve insulin sensitivity (20). However, there have been conflicting studies evaluating the association between palmitoleic acid and human health (25, 26). In a cross-sectional study of obese human subjects, there was no difference in the free fatty acid palmitoleic acid and very low density lipoprotein (VLDL)

palmitoleic acid concentrations between insulin-resistant or insulin-sensitive groups (26).

Insulin-resistant and –sensitive subjects were matched for BMI, age, and % body fat and compared using parametric tests of grouped data (26). There was a high degree of individual variability in palmitoleic acid levels, which highlights the importance of a larger sample size for repeatability (26). This cross-sectional study contrasted with a prospective cohort study using 100 subjects comparing insulin sensitivity and plasma free fatty acid levels using linear regression (25, 26). The subjects in the cross-sectional study had a much greater average BMI than subjects in the study by Stefan et al (36.1 vs. 29 kg/m², respectively). In contrast to the cross-sectional study, researchers evaluating the prospective cohort found that free fatty acid palmitoleic acid levels (C16:1n7) correlated strongly with insulin sensitivity ($p=0.005$) (25).

Following 9 months of increased exercise, lower fat diet (<30%), lower saturated fat intake (<10%), and an increase in fiber, insulin sensitivity and free fatty acid palmitoleic acid increased ($p<0.0001$) (25). Higher initial levels of free fatty acid palmitoleic levels resulted in greater increases in insulin sensitivity with lifestyle intervention (25). If higher levels of palmitoleic acid in plasma free fatty acids do indeed positively influence insulin sensitivity, then it is prudent to assess what, if any, dietary factors influence *de novo* lipogenesis to produce palmitoleic acid and also to consider what other factors of metabolism and energy homeostasis may be affected by higher levels of palmitoleic acid.

Specific Aims

We are utilizing fasting plasma samples collected from a small cross-over study of healthy normal-weight individuals consuming fructose-, glucose-, or aspartame-sweetened beverages while consuming a standard prepared diet.

Specific Aim 1: To assess the effect of different sugar-sweetened beverages with standard diet on *de novo* lipogenesis in the liver versus adipose tissue using a cross-over design. *We hypothesize that during a period of glucose-sweetened beverage consumption, there will be higher levels of palmitoleic acid in plasma free fatty acids, reflecting higher adipose de novo lipogenesis, compared to aspartame. We also hypothesize that during a period of fructose-sweetened beverage consumption; there will be higher levels of palmitoleic acid in plasma phospholipids, reflecting higher hepatic de novo lipogenesis, compared to aspartame. The ratio of palmitoleic acid in phospholipids to free fatty acids will be higher following the diet high in fructose than the diet high in glucose.*

Public Health Impact

Existing data already strongly suggests that fructose intake contributes to obesity-related diseases. Fructose is known to induce hepatic *de novo* lipogenesis, yet may have multiple mechanisms for adverse health effects. Improved insulin sensitivity, glucose tolerance, and decreased hepatic triglycerides in FABP4, 5^{-/-} KO mice correlated with greater metabolic health and higher free fatty acid levels of palmitoleic acid, and reduced ectopic fat deposition (21). If this improvement in metabolic health could be mimicked in humans, this would have

implications in the fight against obesity and metabolic syndrome. One method could be increasing free fatty acid levels of palmitoleic acid, which is achieved through adipocyte rather than hepatic *de novo* lipogenesis.

Improved understanding of factors involved in the pathogenesis of obesity can lead to better public health recommendations. If free fatty acid palmitoleic acid should affect lipid metabolism in the liver, then it may be of great interest to identify dietary factors that differentially affect its concentration. Fructose intake is a popular topic for public health advocates and researchers. Our study may suggest an additional pathway through which fructose versus glucose may differentially affect energy and glucose homeostasis. As important, glucose in the form of mono- or disaccharides is generally seen as a negative component of diets as well, due to the fact that it leads to substantial glycemia and insulinemia. If, however, an increase in peripheral glucose concentrations was shown to increase *de novo* lipogenesis in adipose tissue, associated with an increased free fatty acid palmitoleic acid content, increased hepatic β -oxidation, decreased hepatic lipogenesis, and increased energy expenditure, then this would add a new dimension to our understanding of the health effects of dietary glucose and the resulting glycemia that thus far has not been appreciated.

If our hypothesis is correct, then there are ramifications for public health policy possibly in recommending glucose intake over fructose intake. Of even greater importance, however, is that our study may improve our understanding of the relationship between diet composition and the etiology of obesity and associated metabolic disease.

Chapter 2: Introduction

Over one third of American adults are obese, defined by having a body mass index greater than 30 kg/m^2 , and this number continues to rise, creating a public mandate to better understand disease etiology and find feasible solutions to slow or even reverse this disturbing trend (2). Obesity increases an individual's risk for developing type 2 diabetes, fatty liver, cardiovascular disease, some types of cancer, and a comprehensive range of symptoms comprising metabolic syndrome (9, 10, 27). The current nutritional environment promotes obesity through calorie-dense, nutrient-poor, affordable foods. Sugar-sweetened beverages are one food choice that can contribute significantly to added sugar and caloric intake (5). Sugar-sweetened beverages are typically sweetened with either sucrose, which is 50% fructose and 50% glucose, or with high fructose corn syrup, usually a mixture of 55% fructose and 45% glucose. Over the past forty years, added sugar consumption has risen dramatically, primarily in the form of sweetened beverages (4). This dietary trend contributes at least partially to obesity through excess calories and perhaps through additional metabolic mechanisms (5).

Extra calories from sugar-sweetened beverages lead to weight gain, but fructose from added sugars has additional effects, independently increasing risk for type 2 diabetes, metabolic syndrome, and coronary heart disease (9-12, 28). Short-term high fructose consumption, compared to high glucose consumption, (30% of calories for 1 day) led to lower plasma glucose and insulin levels and increases hepatic *de novo* lipogenesis, in turn raising triglycerides and decreasing HDL levels (1). Most people, however, do not consume fructose

solely in the short term, but as part of their regular diet over an extended period of time. Long-term fructose consumption (25% of calories for 10 weeks) resulted in impaired insulin sensitivity, indicated by higher fasting glucose and insulin concentrations after 10 weeks (18).

In evaluating differences in metabolism between fructose and glucose, it is clear that fructose has a number of deleterious effects, including increased cholesterol and triglyceride levels and decreased insulin sensitivity (1, 18). In addition to implications on hormones and appetite-regulation, fructose and glucose may have differing effects on *de novo* lipogenesis in liver and adipose tissue, both of which may be important determinants of metabolic health. Fructose, due to its unique metabolic pathway, induces *de novo* lipogenesis in the liver, likely contributing to fatty liver and higher cholesterol levels following high fructose consumption (5). Glucose, in contrast, normally induces an insulin response and has a higher degree of metabolism in adipose tissue and muscle. The balance between adipose tissue and hepatic *de novo* lipogenesis has implications for metabolic health, and dietary influence could provide insight into the obesity epidemic and related health problems.

Two pivotal studies evaluated differences in adipose and hepatic *de novo* lipogenesis using double knockout mice of fatty acid binding protein 4 and 5 (FABP^{-/-} KO) (20, 21). The FABP^{-/-} KO mice were resistant to diet-induced obesity and had superior insulin sensitivity and less fat accumulation in the liver (20, 21). FABP^{-/-} KO mice had more β -oxidation in the liver, increased energy expenditure, better insulin sensitivity, and higher resting energy expenditure, all indicators of better metabolic health. An additional result of interest was FABP^{-/-} KO mice

had higher levels of palmitoleic acid in adipose tissue and in the free fatty acid fraction of blood plasma. Palmitoleic acid is primarily generated through *de novo* lipogenesis, making it a good biomarker of *de novo* lipogenesis. An increase in palmitoleic acid in the FABP^{-/-} KO mice suggested an increase in adipose tissue *de novo* lipogenesis associated with their improved metabolic health. Adipose tissue *de novo* lipogenesis has potential implications for energy homeostasis and metabolic health in mice, and is also of interest as applied to human subjects.

Considering the potential role of palmitoleic acid on metabolic health, it is interesting to evaluate different dietary factors in altering the balance of hepatic versus adipose tissue *de novo* lipogenesis in humans. It is known that fructose triggers hepatic *de novo* lipogenesis; less is known about the effect of dietary glucose on adipose tissue versus hepatic *de novo* lipogenesis. Palmitoleic acid may be used as an indicator of differential *de novo* lipogenesis and offer some explanation to glucose and fructose's effects on *de novo* lipogenesis.

Better understanding of factors involved in the pathogenesis of obesity can lead to improved interventions of diet and health. Added sugars comprise a substantial part of many Americans' diets without complete understanding of their effect on metabolic health. If fructose does strongly affect metabolic health and energy homeostasis, this could be an influence to modify current policy and guidelines with the recommendation to favor glucose or starch intake over fructose intake. If glucose intake is seen to increase free fatty acid palmitoleic acid content and positively influence metabolic health relative to equal amounts of fructose, this would add new understanding to health effects of glycemia. Ultimately, our study

may improve our understanding of the relationship between diet composition and the etiology of obesity and associated metabolic disease.

Chapter 3: Methods

Study Design

This was a double-blind controlled randomized cross-over design diet study. The study's primary aim was to evaluate caloric intake, hormonal, and metabolic effects of sweetened beverages on healthy young adults. Each subject participated in three diet periods where solid food remained the same for each of the three periods in addition to a differently sweetened beverage for each period. The beverage was sweetened with either fructose, glucose, or a non-caloric sweetener (aspartame).

Subjects were randomized to receive diets with beverages sweetened with fructose, glucose, or aspartame in three 8-day periods. The study design is shown in Figure 3.1. On day 9 of each period, subjects were admitted to the Clinical Research Center (CRC) of the University of Washington for procedures outlined below. Each diet period was separated by a 20-day washout period so female subjects were on the same day of their menstrual cycle. During the wash-out period, subjects resumed their usual diet. Subjects were asked to keep physical activity constant throughout the study.

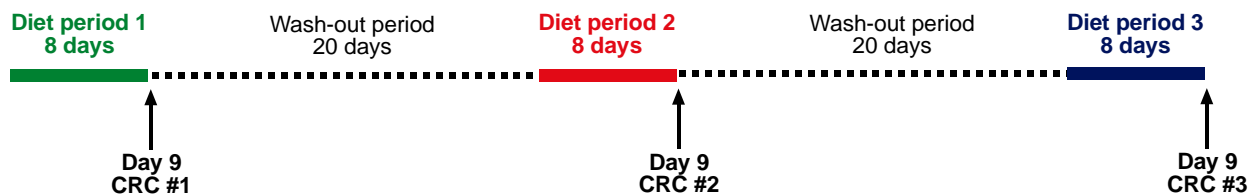


Figure 3.1 Design of the study. Three randomized 8-day diet periods separated by 20 wash-out days for each subject. Day 9 of each diet period includes an admission to the CRC at UW.

Subjects

Between September 2009 and June 2011, 157 volunteers were screened over the phone in response to study flyers and newspaper ads, as shown in Appendix 1. Volunteers were screened over the phone using inclusion and exclusion criteria outlined in Table 3.1

Table 3.1 Inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none">• Age 18-25 years at time of enrollment• BMI: 20-25 kg/m²• Weight stable within 5 pounds for 6 months prior to entering the study, and within 10 pounds of lifetime maximum weight• Ability to be admitted for 5 hours to the CRC at the UW Med Center on three occasions• Willing to consume only food and beverages provided by the Nutrition Research Kitchen of the UW CRC for three 8-day periods• Ability to provide informed written consent	<ul style="list-style-type: none">• Use of prescription medications for a chronic medical condition, recreational drugs or tobacco products• Presence of fructose malabsorption or hereditary fructose intolerance• Use of antibiotic drugs within 3 months of enrollment into the study• Regular intense exercise (>3 hours per week)• Vegetarian/extreme dietary preferences• Alcohol consumption of more than 2 drinks per day• Frequent past attempts at weight loss• Current dieting or weight control program

Following phone screening, 31 volunteers were given study consent documents and invited to a physical screening visit at the UW Clinical Research Center (CRC), where basic anthropometric data were collected, an exercise questionnaire was completed, and a fructose-tolerance test was administered. Subjects arrived at 8:00 a.m. after a 12-hour overnight fast to obtain fasting blood samples and to undergo the fructose-tolerance test. The blood sample was taken for measuring plasma lipids, glucose, creatinine, plasma glutamic-oxaloacetic transaminase (SGOT), and thyroid stimulating hormone (TSH). Subjects were healthy normal-weight volunteers who agreed to participate in the study, passed all screening tests, and provided informed consent. Following the fasting blood draw and consent procedures, subjects were given one fructose-sweetened beverage containing 6.25% of estimated daily

calorie intake and the fructose-tolerance test was administered. The fructose-tolerance test uses a hand-held device that measures hydrogen exhaled to determine undiagnosed fructose malabsorption; the protocol is shown in Appendix 2. An increase in hydrogen indicates fructose malabsorption and results in exclusion from the study if subjects hydrogen consumption exceeds 20 ppm over baseline for two time points. Hydrogen levels were measured at 0, 30, 60, 90, 120, 150, and 180 minutes (Micro H₂ Meter, Lewiston, ME) (29). Upon successful completion of the CRC screening visit, 10 volunteers were enrolled in the study. Many subjects were not enrolled in the study due to fructose intolerance. One enrolled subject was subsequently dropped due to changes in physical activity after completing two diet periods. This subject's data was excluded from analysis. Nine subjects completed the study.

Diets

All meals were provided to subjects during each 8-day study diet period. Foods were prepared at the Nutrition Research Kitchen of the UW CRC and subjects picked up foods 3 or 4 times during each 8 day period. Solid foods were provided in excess (125%) of estimated calorie requirements calculated by incorporating activity level and the Mifflin-St. Jeor formula, which takes into account subjects height, weight, age, and activity level. Activity level was estimated using the validated Blair physical activity questionnaire (30). Subjects participated in all three arms of the study in random order. Men and women were randomized separately. All meals were *ad libitum* consumption with uneaten food returned to the Research Kitchen for weighing. Subjects were required to consume all beverages provided. Beverages were provided at 25% of estimated calorie intake for the glucose- and fructose-sweetened beverage

phases. During the aspartame phase the same volume and sweetness of beverages were provided.

Clinic Visits

On day 9 of the study diet periods, participants were admitted to the UW CRC at 6:30 a.m. after a 10-hour fast. Weight and vital signs were taken. Subjects' resting metabolic rate was assessed using indirect calorimetry (Parvo Medics True One 2400, Sandy, UT) after 30 minutes of rest. At 8:00 a.m., fasting blood samples were drawn, collected in EDTA tubes and placed on ice. An additional fasting sample was collected with THL for free fatty acid measurements. Samples were then immediately centrifuged for 10 minutes at 3000 rpm at 4°C. Following the fasting blood draw, subjects consumed a standardized breakfast followed by six post-prandial blood samples over the course of 2.5 hours (time points 0, 15, 30, 60, 90, 120 min). At each blood draw the subject also completed an appetite questionnaire and was given an *ad libitum* meal 120 minutes after the standardized breakfast. Blood samples were immediately spun and plasma was aliquoted and frozen at -70°C.

Laboratory Analyses

Subjects were asked to return all uneaten food to the Nutrition Research Kitchen for assessment of mean energy intake using ProNessy Software. Palmitoleic acid was measured using gas chromatography in the phospholipid and free fatty acid fraction of plasma in the fasting sample at the Fred Hutchinson Cancer Research Center Biomarker Laboratory. Total lipids are extracted from serum using the method of Folch (31), and phospholipids & free fatty

acids separated from other lipids by one dimensional thin layer chromatography (32). Fatty acid methyl ester (FAME) samples are prepared by direct transesterification using the method of Lepage (33) and separated using gas chromatography (Agilent 5890 Gas Chromatograph FID detector; Supelco fused silica 100m capillary column SP-2560; initial 160°C for 16 min, ramp 3.0°C/min to 240°C, hold for 15 min). Fatty acid composition is expressed as weight percentage of the total. The assay generates data on 46 fatty acids. CV were <5% for most fatty acids except for the ones with very low abundance.

Fasting plasma concentrations of glucose, insulin, triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol were performed on a Hitachi 917 autoanalyzer (Boehringer Mannheim, Mannheim, Germany) at Northwest Lipid Research Labs. Intra-assay CV for all endpoints measured at NWLRL were <2.5%.

Statistical Analyses

Data from subjects was analyzed with the statistical software program SPSS (version 16; SPSS Inc. Chicago, IL). The level of significance was set at $\alpha=0.05$. Repeated measures analysis of variance (RM-ANOVA) was used to compare levels of palmitoleic acid in plasma phospholipids and free fatty acids for each diet period. If RM-ANOVA was not appropriate due to the assumption of normally distributed residuals not being met, then the non-parametric Friedman test was used. *Post hoc* analysis was done using paired t-tests for normally distributed standardized residuals. For data that was not normally distributed, the Wilcoxon signed rank test was used in *post hoc* analysis. For *post hoc* analysis, p-values were multiplied

by 3 to adjust according to the Bonferroni method. To evaluate the relationship between palmitoleic acid concentrations in the phospholipid and free fatty acid levels on the one hand, and markers of metabolic health, such as fasting triglycerides and resting energy expenditure on the other, multiple linear regression was used, taking into account subject number and diet period.

Chapter 4: Results

Subject Characteristics

Ten subjects were enrolled into the study and nine completed all three diet phases and day nine CRC admissions. One subject was excluded due changes in physical activity levels between diet periods. Subject baseline characteristics are outlined in Table 4.1. Subjects were all young and normal weight individuals.

	Minimum	Maximum	Mean	Std. Deviation
Age (yr)	19.0	24.0	20.9	1.97
Weight (kg)	56.4	76.7	66.0	6.47
BMI (kg/m ²)	20.1	24.1	22.7	1.27
Fasting serum triglycerides (mg/dl)	38.0	145	76.4	34.3
Fasting serum total cholesterol (mg/dl)	126	185	150	22.8
Fasting serum HDL (mg/dl)	29.0	61.0	46.9	12.1
Fasting serum LDL (mg/dl)	65.0	129	88.6	21.4

Energy Intake

Mean energy intake varied significantly between the sugar-sweetened diet periods and the aspartame-sweetened diet period ($p=0.002$), as shown in Table 4.2. Daily calorie intake during glucose- and fructose-sweetened beverage diet periods were significantly higher than during the aspartame-sweetened beverage diet period, according to *post hoc* paired t-tests.

Expressed as a percentage of daily energy intake during the aspartame diet phase, diet periods with glucose- and fructose-sweetened beverages were not significantly different from each other ($p=0.085$, adjusted $p=0.255$). Percentages of caloric intake were significantly statistically different between the fructose- and aspartame-sweetened beverage diet periods ($p=0.001$, adjusted $p=0.003$). Percentages of caloric intake were also significantly statistically different between the glucose- and aspartame-sweetened beverage diet periods ($p=0.001$, adjusted $p=0.003$). Protein, carbohydrate, and fat intake, expressed as a percentage of total calories consumed, were all statistically significantly different between the aspartame diet period and the glucose or fructose diet periods.

Table 4.2 Effect of sweetened beverage consumption on daily energy intake, diet composition, and daily energy as a percentage of aspartame phase daily energy intake*, $n=9$

	Glucose	Fructose	Aspartame	RM-ANOVA
Daily energy intake (kcal)	2629±682 ^z	2698±607 ^z	2307±651	F=17.8, $p=0.002$
Daily energy intake (% of aspartame daily energy)	115.01±8.43 ^z	119.09±11.3 ^z	100±0	F=13.0, $p=0.004$
Protein intake (% total)	13.8±0.313 ^z	13.5±0.267 ^z	16.4±0.183	F=187, $p<0.001$
Carbohydrate intake (% total)	62.3±1.15 ^z	62.4±0.981 ^z	54.8±1.146	F=233, $p<0.001$
Fat intake (% total)	24.0±1.00 ^z	24.1±1.01 ^z	28.9±1.076	F=92.7, $p<0.001$

*Diet periods not sharing the same superscript symbol are significantly different from each other in *post hoc* tests, $p<0.05$ adjusted according to the Bonferroni method.

Palmitoleic Acid

Palmitoleic acid levels, measured as a percentage of total fatty acids, were analyzed from a fasting blood sample on day nine of each diet period. Palmitoleic acid (C16: 1n-7 cis)

was measured in both the free fatty acid (FFA) and the phospholipid (PL) fraction of blood plasma. There was no significant difference in plasma FFA C16: 1n-7 cis in any of the diet periods ($p=0.740$), as shown in Table 4.3. There was a significant difference in the plasma PL C16: 1n-7 cis fraction. In *post hoc* analysis, there was not a significant difference between PL C16:1n-7 cis content in fructose or glucose phases ($p=0.103$, adjusted $p=0.309$). There was a significant difference between the fructose and aspartame diet periods ($p=0.005$, adjusted $p=0.015$). There was not a significant difference between the glucose and aspartame diet periods ($p=0.364$, adjusted $p=1.092$).

Table 4.3 Effect of sweetened beverage consumption on palmitoleic acid levels in plasma free fatty acids and plasma phospholipids, expressed as a % of total fatty acids*, n=9

	Glucose	Fructose	Aspartame	RM-ANOVA
Plasma FFA C16: 1n-7 cis	3.21±0.90	3.22±1.23	3.32±0.729	F=0.315, $p=0.740$
Plasma PL C16: 1n-7 cis	0.40±0.14 ^{xy}	0.48±0.145 ^z	0.37±0.091 ^y	F=7.24, $p=0.020$
				Friedman's Test
Ratio of C16: 1n-7 cis in PL/FFA	0.12±0.02 ^{xy}	0.17±0.093 ^z	0.11±0.016 ^y	$p=0.018$

*Diet periods not sharing the same superscript symbol are significantly different from each other in *post hoc* tests, $p<0.05$ adjusted according to the Bonferroni method.

When evaluating the ratio of C16: 1n-7 cis in PL/FFA fraction of blood plasma, there was a significant difference between diet periods. Because the ratio of C16: 1n-7 cis in PL/FFA was not normally distributed, Friedman's test was used. There was a statistically significant difference between the ratio of palmitoleic acid in the phospholipid fraction relative to the free fatty acid fraction between diet periods ($p=0.018$). In *post hoc* analysis, the Wilcoxon signed

rank test was used and there was a significant difference between the ratio of PL/FFA of C16:1n-7 cis of the aspartame and fructose-sweetened beverage diet periods ($p=0.015$, adjusted $p=0.045$). There was not a significant difference in the ratios comparing glucose or fructose-sweetened beverage diet periods ($p=0.086$, adjusted $p=0.258$), or the glucose or aspartame-sweetened beverage diet periods ($p=0.066$, adjusted $p=0.198$).

Linear regression analysis was performed to evaluate the relationship between resting energy expenditure and palmitoleic acid in the phospholipid fraction. Resting energy expenditure was not significantly associated with diet period ($p=0.080$) or subject number ($p=0.599$), but was significantly associated with palmitoleic acid levels in the phospholipid fraction ($p<0.001$), as shown in Table 4.4. The subject number and diet period were potentially important to adjust for, as we included all three time points from each subject into the multiple linear regression analysis. When palmitoleic acid in the free fatty acid fraction was added, significance declined for the phospholipid fraction, which is shown in Appendix 3. When palmitoleic acid in the free fatty acid was added to the model, it was not significant.

Table 4.4 Effect of diet period, subject number, and PL 16:1n-7 cis on resting energy expenditure (REE), n=9

	Unstandardized coefficient	Standard error	Standardized coefficient	t	P
REE	2274	211		10.8	0.000
Subject number	-2.28	4.28	-0.079	-0.533	0.599
Diet period	-85.195	46.5	-0.293	-1.83	0.080
PL 16:1n-7 cis	-1383	299	-0.740	-4.63	<0.001

Linear regression analysis was used to evaluate the association between subject, diet period, and palmitoleic acid in the phospholipid and free fatty acids of plasma on fasting triglyceride levels in subjects, as shown in Table 4.5. There were no significant associations between either subject ($p=0.376$) or diet period (0.796) and fasting triglyceride levels. There was a significant association between both palmitoleic acid in the phospholipid fraction ($p=0.022$) and the free fatty acid fraction of plasma ($p=0.011$). The β -coefficient for palmitoleic in the phospholipids was 2.47 and for the free fatty acid fraction was -2.76.

Table 4.5 Effect of subject, diet period, PL 16:1n-7 cis and FFA 16: 1n-7 cis on fasting triglycerides, n=9

	Unstandardized coefficient	Standard error	Standardized coefficient	t	P
Triglycerides	118	27.7		4.26	<0.000
Subject number	-0.580	0.642	-0.165	-0.904	0.376
Diet period	-1.690	6.46	-0.047	-0.262	0.796
PL 16:1n-7 cis	125	50.7	0.545	2.47	0.022
FFA 16:1n-7 cis	-19.9	7.22	-0.629	-2.76	0.011

Chapter 5: Discussion

The overall objective of this study was to evaluate caloric intake, hormonal, and metabolic effects of sweetened beverages in healthy young adult volunteers. The specific aim was to evaluate the differential effect of sweetened beverages on palmitoleic acid in either free fatty acid or phospholipid fractions in fasting blood.

Energy Intake

Subjects consumed an additional 25% of daily calorie intake as sweetened beverages, which resulted in greater overall calorie consumption during fructose- and glucose-sweetened beverage diet periods. Calorie intake and diet composition were statistically different between glucose- and aspartame-sweetened diet periods and between fructose- and aspartame-sweetened diet periods but not between glucose- and fructose-sweetened diet periods. Subjects did not fully compensate for calories in beverages by eating less solid food for either glucose- or fructose-sweetened beverage diet periods.

Palmitoleic Acid

In the fasting free fatty acid fraction of plasma, there was no significant difference between diet groups in palmitoleic acid levels. This is a surprising result, given that we expected glucose to induce higher levels of palmitoleic acid in the free fatty acid fraction due to a hypothesized increase in adipose *de novo* lipogenesis. However, our result could be explained by the use of a fasting blood sample in analysis. The blood samples used in this study to

evaluate fat composition in blood plasma free fatty acids and phospholipids represent the body in a fasting state, which is a state of lipolysis, not a state of anabolic activity. Palmitoleic acid that originates in adipose tissue may be important in metabolic function as indicated by mouse studies, yet we saw no difference in a fasting blood sample in these nine individuals. Palmitoleic acid is primarily produced through *de novo* lipogenesis, which makes it a good marker for *de novo* lipogenesis.

In support of our hypothesis, palmitoleic acid plasma phospholipid levels were higher during the fructose-sweetened diet period compared to the aspartame-sweetened diet period, which indicates an increase in hepatic *de novo* lipogenesis induced by fructose. While palmitoleic acid levels in the phospholipid fraction of plasma during the glucose diet period was not significantly different from the fructose diet period, it was lower, indicating a trend for increased hepatic *de novo* lipogenesis. During the aspartame-sweetened diet period the levels of C16: 1n-7 cis in phospholipids were the lowest. A larger sample size resulting in increased power would provide valuable insight into this and other trends as sample size increased. The unadjusted p-values for the ratio of PL/FFA of C16: 1n-7 cis between fructose and glucose-sweetened beverage diet periods and between glucose and aspartame-sweetened diet periods approached significance and it would be valuable to increase sample size and evaluate these ratios again.

In multiple linear regression analysis of palmitoleic acid on resting energy expenditure and fasting triglycerides, subject number and diet period were included in the analysis to

account for changes in beverages and also any differences between individuals. Even though subject number and diet period were not significant in either analysis, we felt it was important to include these factors. Resting energy expenditure and palmitoleic acid in the phospholipid fraction were significantly associated, which suggests that hepatic *de novo* lipogenesis could play an important metabolic role as suggested by mice studies. A higher palmitoleic acid level in phospholipids correlates with a lower resting energy expenditure, which aligns with predictions that hepatic *de novo* lipogenesis may have negative metabolic effects.

Fasting triglycerides were significantly associated both with palmitoleic acid in the phospholipid and free fatty acid fractions in multiple linear regression analysis. Interestingly, the apparent relationship between palmitoleic acid in phospholipids and free fatty acids and fasting triglycerides is as predicted and supports a beneficial metabolic effect of adipose tissue *de novo* lipogenesis or free fatty acid palmitoleic acid and a detrimental effect of hepatic *de novo* lipogenesis or phospholipid palmitoleic acid. According to our results, higher palmitoleic acid free fatty acids are significantly associated with lower fasting triglycerides, while higher palmitoleic acid phospholipid levels are significantly associated with higher fasting triglycerides.

Study Strengths and Limitations

While a cross-over study design can allow for smaller numbers of participants due to each subject acting as their own controls, nine may be too small to see some effects of sweetened beverages on metabolic outcomes. While adjusting for multiple comparisons did not change the significance of any p-values, there were trends that could potentially be

influenced by a larger sample size. The randomized double-blind design is a notable strength of the study design. Measurements of fructose tolerance by breath test and two screening steps helped ensure proper fructose absorption by subjects. Previous studies by Stanhope et al. and by Teff et al. did not include fructose tolerance testing. Diet preparation by the CRC Research Kitchen ensured identical foods and accurate caloric analysis of diets.

This study evaluated the effects of fructose-, glucose-, and aspartame-sweetened beverages on young healthy subjects. A limitation of this study might have been the study of isolated monosaccharides in a healthy population while the general population consumes beverages generally sweetened with sucrose or HFCS. This was an intentional decision in this study to evaluate monosaccharide's effects on health, yet does not necessarily directly translate to commonly available beverages. However, it is of interest to evaluate monosaccharide's metabolic effects to make stronger recommendations about the health effects of high fructose versus high glucose beverages, or to recommend a change in composition of sugars used in processed foods. In addition, the majority of the population is overweight or obese and may metabolize sugars differently than a young healthy study population

In summary, despite the small sample size and fasting blood sample, we found a significant difference between palmitoleic acid levels in the phospholipid fraction in fructose- and aspartame-sweetened beverages. The significant associations between resting energy expenditure and palmitoleic acid from hepatic *de novo* lipogenesis suggest a potential

detriment to energy expenditure from increased hepatic *de novo* lipogenesis. Similarly, the associations between fasting triglycerides and palmitoleic acid suggest a metabolic benefit from adipose *de novo* lipogenesis and a detriment from hepatic *de novo* lipogenesis. Research into dietary influences on the balance between hepatic and adipose *de novo* lipogenesis offers further insight into the etiology of obesity-related diseases and could provide a stronger basis for public health recommendations to reduce fructose consumption.

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Appendix 1: Study Recruitment Advertisement

Do you like soda pop?

Are you interested in the health effects of soda drinks?

Then you might want to participate in a study that we are doing at the University of Washington and Fred Hutchinson Cancer Research Center.

We are looking for men and women who are:

- 18 – 25 years old;
- Of normal body weight;
- Willing to drink 30-60 ounces of sweetened beverages per day for three periods of 8 days each;
- Eat all meals and snacks exclusively from food provided by the University of Washington Nutrition Research Kitchen for these 3 periods of 8 days each;
- Able to pick up this food three times weekly during weekday business hours at the University of Washington;
- Able to be admitted to the University of Washington General Clinical Research Center for 5 hours on three occasions over 3 months.

Benefits to volunteers:

- You will receive all food and beverages from us for a total of 24 days AT NO COST.
- You will be reimbursed for completing the study.
- You will learn about how your metabolism is affected by different sweetened beverages.

Fred Hutchinson Cancer Research Center

Public Health Sciences Division

For more information call: 206-667-6020

Appendix 2: Hydrogen Breath Test Procedure

Conduct of hydrogen breath test to detect fructose malabsorption (modified from Keller et al.; Z. Gastroenterol 2005; 43: 1071-90)

Prepare subjects:

- Ask subject if they suffer from hereditary fructose intolerance.
- Ask subjects not to take antibiotics in the week before the test (screening visit).
- Ask subjects to eat a low-fiber diet on the day before the test (screening visit).
- Ask subjects to eat their last meal before 8 PM on the evening prior to their screening visit (all screening visits will be scheduled in the morning).
- Ask subject to use an antibacterial mouthwash before the procedure; this will eliminate the chance that fructose might already be fermented in the mouth by oral bacteria, leading to potential H₂ formation.

Perform test:

- Make sure H₂ analyzer is calibrated.
- For all H₂ measurements, ask subject specifically to exhale slowly; if any result doesn't seem plausible, repeat and mention again to exhale slowly.
- Measure H₂:
 - If this baseline value is <10 PPM, perform the test as planned.
 - If baseline value is >30 PPM, re-schedule to perform test on a different day.
 - If baseline value is between 10 and 30 PPM, repeat the antibacterial mouthwash and the measurement to try to get it under 10 PPM. If still between 10 and 30 PPM, wait 10 minutes and repeat the test a third time. Proceed with the test if baseline after 3 measurements is <30 PPM.
 - If you have to re-schedule, remind subject to adhere to a low-fiber diet on the day prior to the test, and to remain fasting for at least 12 hours.
- Provide subjects with one serving of the fructose-sweetened beverage. The amount of the beverage provided will be adjusted to provide 6.25% of estimated total energy intake, as this is equivalent to one of the four servings subjects will be consuming daily during the study diet periods. Subjects will be asked to drink the entire beverage within 15 minutes.
- Measure H₂ again 0, 30, 60, 90, 120, 150, and 180 minutes after the subject has finished the meal.
- Make notes if subject complains of any clinical symptoms such as abdominal cramps during the test.
- After the 180 minute test, ask the subject to complete a questionnaire to assess any clinical gastrointestinal symptoms that could be related to fructose malabsorption.
- Diagnosis of fructose malabsorption is made if peak postprandial hydrogen concentration exceeds 20 PPM above baseline at two or more time points.

Appendix 3: Effect of subject, diet period, PL 16:1n-7 cis and FFA 16:1n-7 cis on resting energy expenditure

Table 4.6 Effect of subject, diet period, PL 16:1n-7 cis and FFA 16: 1n-7 cis on resting energy expenditure

	Unstandardized	Standard error	Standardized	t	P
	coefficient		coefficient		
REE	2247	215		10.5	<0.001
Subject number	-1.53	5.01	-0.053	-0.306	0.762
Diet period	-80.0	50.4	-0.275	-1.59	0.127
PL 16:1n-7 cis	-1307	396	-0.699	-3.30	0.003
FFA 16:1n-7 cis	-17.2	56.4	-0.067	-0.305	0.764