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Ran Jin Date

Consumption of Fructose and Markers of Cardiovascular Disease Risk among US Adolescents with Nonalcoholic Fatty Liver Disease

By

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Doctor of Philosophy
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Consumption of Fructose and Markers of Cardiovascular Disease Risk among US Adolescents with Nonalcoholic Fatty Liver Disease

By

Ran Jin
M.D., Nanjing Medical University, 2006

Advisor: Miriam Vos, MD, MSPH

An abstract of
A dissertation submitted to the Faculty of the
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Abstract

Consumption of Fructose and Markers of Cardiovascular Disease Risk among US Adolescents with Nonalcoholic Fatty Liver Disease

By Ran Jin

Nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome, is the most common cause of chronic liver disease in western countries. Adults with NAFLD have increased morbidity and mortality rate from cardiovascular disease (CVD), and more importantly, adolescents with NAFLD demonstrate early signs of subclinical atherosclerosis. Dietary fructose has been proposed as a culprit in the development and progression of NAFLD, and fructose-induced metabolic abnormalities are likely to be associated with increased CVD risk seen in NAFLD. Adolescents are reported to have the highest fructose intake (primarily from fructose-sweetened beverages); however, little is known about the consumption of fructose and markers of CVD risk among U.S. adolescents with NAFLD.

In the body of research for this dissertation, we hypothesized that fructose could be a modifiable dietary factor to ameliorate CVD risk in NAFLD. Thus we implemented a series of studies to 1) determine whether fructose consumption could acutely alter/exacerbate the lipid profile, glycemic status, and other CVD risk factors in adolescents with NAFLD, 2) to determine if fructose restriction could improve hepatic steatosis, lipids, insulin resistance, and other markers related to CVD risk in adolescents with NAFLD, and 3) to evaluate whether endotoxin could be a mediator through which fructose induces NAFLD and associated CVD risk in adolescents.

Through these studies, we found that in adolescents with NAFLD, fructose acutely induced the postprandial dyslipidemia (a high-triglyceride, low-high density lipoprotein pattern), an important risk factor in the pathogenesis of atherosclerosis. In addition, by performing a calorie-matched, randomized controlled trial, we found that fructose reduction for 4 weeks resulted in an improved cardiometabolic profile, including increased insulin sensitivity, reduced inflammation and LDL oxidation. This improvement could be possibly mediated by the fructose-induced endotoxemia. Further research is needed to evaluate potential benefits of fructose restriction in a longer intervention period, and to fully investigate the underlying biological mechanisms of fructose in the early atherosclerotic lesions seen in pediatric NAFLD.

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Chapter I. Introduction

Nonalcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease in the U.S. adolescents over the past two decades[1]. NAFLD can progress to nonalcoholic steatohepatitis (NASH) and end-stage liver disease, but the global health risk of NAFLD is not confined to the liver. Longitudinal data suggest that there is increased risk of cardiovascular disease (CVD) in adults with NAFLD[2,3]. Although the pediatric population with NAFLD has not been followed long enough to examine whether they have increased cardiovascular event in their later life, evidence from clinical reports indicate that adolescents with NAFLD already manifest early signs of atherosclerotic lesions[4,5]. However, the underlying mechanisms are not yet fully investigated and therapeutic strategies have not been well established.

Fructose has been proposed as a critical risk factor in the development and progression of NAFLD. Strong evidence from animal experiments and short-term feeding studies in adults suggests that fructose consumption, in a hypercaloric setting, may promote *de novo* lipogenesis, hepatic fat accumulation, visceral adiposity, hypertriglyceridemia, and insulin resistance[6-8]. In addition, sustained ingestion of fructose has been reported to increase plasma concentrations of fasting small dense LDL and oxidized LDL[7]. These changes induced by fructose are associated with increased risk of CVD. In particular, patients with NAFLD already have dysregulated lipid metabolism[9], and thus they may have decreased tolerance of this lipogenic sugar, fructose, which is metabolized through similar mechanisms.

Adolescents are reported to have the largest consumption of fructose, accounting for approximately 12% of their total calories[10]. Because more than 75% of fructose in a typical westernized diet is from added sources, primarily sugar-sweetened beverages[10], it is practical that fructose might be a readily modifiable dietary factor to manage NAFLD and its associated cardiovascular risk. In spite of the fact that adolescents have a high prevalence of NAFLD[1] and possibly a more aggressive form of the disease[11], previous studies on the effect of fructose on NAFLD in this age group are very limited. Therefore, the body of research for this dissertation focused on the pediatric population and was designed to answer the following questions:

1). Does fructose consumption acutely alter/exacerbate the lipid profile, glycemic status, and other cardiovascular risk factors in adolescents with NAFLD?

2). Does fructose restriction improve hepatic steotosis, lipids, insulin resistance, and other markers related to CVD risk in adolescents with NAFLD?

3). Could endotoxin be a possible mediator through which fructose induces CVD risk seen in adolescents with NAFLD?

This document provides, in Chapter II, a literature review on NAFLD including its prevalence, histological findings, etiology, therapeutics, and its associated cardiovascular risk. In addition, Chapter II also provides the review of the literature on the consumption of dietary fructose, fructose metabolism, and its consequently adverse health outcomes. Chapter III is a summary of analyses performed to determine whether short-term fructose feeding alters lipid profile and other metabolic markers related to CVD risk in adolescent

with NAFLD. Chapter IV states the evidence that determines if 4-week fructose reduction improves cardiometabolic profile in adolescents with NAFLD. In Chapter V, preliminary data are generated to describe whether endotoxin could be a mediator in the management of fructose-induced CVD risk in pediatric NAFLD. Chapter VI summarizes findings of this research project, and proposes possible future directions and significant implications of public health.

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Chapter II. Literature Review

II.1 Nonalcoholic Fatty Liver Disease (NAFLD)

1.1 Definition

Nonalcoholic fatty liver disease (NAFLD) is an umbrella term to describe a wide range of related disorders including simple steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. Hepatic steatosis, the earliest stage of NAFLD, is characterized by the deposition of triglyceride in more than 5% of hepatocytes, with insignificant alcohol consumption (no more than 2 alcoholic drinks per day for men and 1 for women)[1]. Hepatic steatosis can progress to NASH, which is distinguished from simple steatosis by the presence of hepatocyte injury and inflammation, with or without fibrosis. Approximately 10-29% of individuals with NASH can progress to cirrhosis within 10 years[2], a condition in which hepatocytes are replaced by scar tissue. Individuals with NASH-induced cirrhosis have increased risk of hepatocellular carcinoma, a terminal condition that needs liver transplantation.

The traditional "two-hit" theory has been utilized to describe the development and progression of NAFLD. The "first hit" involves an imbalance in free fatty acid (FFA) metabolism[3]. Excess flux of FFA from peripheral tissues to the liver is increased, possibly due to the resistance to insulin-mediated suppression of lipolysis[4], thus leading to accumulation of triglyceride droplets within the hepatocytes. As a consequence, the steatotic liver is then made more vulnerable to additional insults, the so-called "second hit", which is oxidative stress and inflammation that result from efforts to compensate for

altered lipid homeostasis[3]. More recently, Jou et al. have proposed the "third hit" as a new component into this theory[5], which is defined as hepatocyte death and lack of repair, and this step is speculated as a significant event driving progression from NASH to cirrhosis.

1.2 Epidemiology

With obesity being an important risk factor universally, NAFLD is now receiving great attention globally and is regarded as a public health issue. The estimation of true prevalence of NAFLD is limited by the fact that individuals with early, and perhaps most, stages of the disease are commonly asymptomatic, and because the gold standard for diagnosing and staging NAFLD is a liver biopsy, which is invasive and not appropriate for screening studies. Therefore, the prevalence estimates vary based on the selection of the specific diagnostic criteria[6] and the available information in a given study population. In the general American population, it is estimated that approximately 3 to 20% have NAFLD based on elevated transaminases, and the estimate varies from 16 to 19% based on ultrasound screening. Autopsy studies suggest that the prevalence of NAFLD ranges from 11 to 36%[1].

Clark et al. examined a nationally representative data on 15,676 adults from the Third National Health and Nutrition Examination Survey (NHANES III) (1988-1994) using serologic tests[7]. They defined NAFLD as having unexplained elevation of aminotransferase levels in the absence of laboratory evidence of hepatitis B or C

infection and iron overload, and a history of alcohol consumption. Elevation of aminotransferases were considered as any value above normal of alanine aminotransferase (ALT) or aspartate aminotransferase (AST) based on NHANES III criteria (ALT > 40 U/L or AST > 37 U/L for men, and ALT or AST > 31 U/L for women). The estimated prevalence of NAFLD was 5.4% based on this definition, corresponding to 9.1 million individuals. In the same study cohort, if including abnormal γ-glutamyltransferase (GGT) level (> 30 U/L) into the definition of elevated aminotransferase[8], the prevalence of presumed NAFLD increased to 23%, representing approximately 31 million people.

Another major large-scale study examining the prevalence of hepatic steatosis in the U.S. population is the Dallas Heart Study which utilized a multiethnic group of 2,287 adults (32.1% white, 48.3% black, and 17.5% Hispanic)[9]. Hepatic steatosis was defined in this study as hepatic triglyceride content greater than 5.5% assessed by the proton nuclear magnetic resonance spectroscopy (^{1}H-MRS). According to this definition, 31% of the study population had hepatic steatosis, with most subjects (79%) having normal levels of serum ALT. This relatively higher prevalence is possibly due to the majority of this study cohort being obese or diabetic, and the prevalence of NAFLD found in the non-obese participants declined to 16.7%.

Prevalence in high risk population

Obesity and insulin resistance are well established risk factors for NAFLD. Machado et al. examined 12 observational studies and demonstrated that, on average, hepatic steatosis in obese patients with the indication of bariatric surgery was 91% (ranging from 85% to 98%)[10]. Other have also described a higher prevalence of NAFLD among patients with type 2 diabetes as compared to nondiabetics, with estimates ranging from 49.0% to 69.5%[11].

Prevalence in pediatric population

The most recent reports from the Center for Disease Control and Prevention (CDC) indicate that 16.9% (corresponding to 12.5 millions) of U.S. youth aged 2-19 years are obese. In parallel with the epidemic of childhood obesity, NAFLD has emerged as the leading cause of chronic liver disease in children. Estimated by Welsh et al., the prevalence of pediatric NAFLD has more than doubled in the past two decades and currently affects approximately 11% of adolescents in the U.S.[12]. An autopsy study reported that 9.6% of the American population aged 2-19 years had NAFLD, and this figure increased to 38% among those who were obese[13]. When further analyzed, the data showed that 17.3% of adolescents aged 15-19 years, compared with 0.7% in the 2-4-year-old range, were affected by NAFLD[13]. Therefore, NAFLD in pediatrics, particularly in adolescents, now presents a major public health crisis and rigorous evaluation is strongly needed.

1.3 Histopathology

In adults, the histopathology of NAFLD has been well described with established criteria for classification and characterization of liver injury and parenchymal changes[14]. The visible steatosis is triglyceride deposition within hepatocytes either as single large droplets (displacing cytoplasmic contents and the nucleus) or smaller, well-circumscribed droplets (admixed with cytoplasmic contents). The primary component of steatosis occurs in perivenular, acinar zone 3 hepatocytes, and the diagnostic criteria for steatohepatitis include steatosis, hepatocellular injury, and lobular inflammation typically occurring with a zone 3 predominance. Hepatocyte injury is most often noted in the form of ballooning, which is characterized by the presence of enlarged, flocculent appearance hepatocytes. In addition, cytoplasmic aggregates referred to as Mallory-Denk Bodies may also be present.

NAFLD in pediatrics may have a different histological presentation[15]. In children with NAFLD, steatosis is frequently observed in periportal, zone 1 hepatocytes and is more commonly moderate or severe than in adults[16]. Pediatric NASH can also have a disparate histological pattern, which is characterized by the typical features of steatosis but a predominance of portal inflammation and fibrosis, in the absence of ballooning degeneration[17]. This distinct pattern of NASH, also known as type 2 NASH, represents the majority of pediatric NASH[17-19], and may simply be a predictor of a subgroup of patients who have more severe form on the spectrum of NASH. By following-up 66 children with diagnosed NAFLD for 20 years, Feldstein et al. demonstrated that children

with NAFLD may develop end-stage liver disease with the consequent need for liver transplantation and may have a shorter survival as compared to the general population[20].

1.4 Etiology

Ethnicity

Recent studies have indicated that individuals from certain ethnicities appear to be predisposed to NAFLD. In a multiethnic, population-based sample, strikingly higher frequency of hepatic steatosis was found in Hispanics (45%) as compared to Whites (33%) and Blacks (24%)[9]. This higher prevalence in Hispanics is possibly attributable to the higher presence of obesity and insulin resistance, but the lower prevalence in Blacks cannot be explained by associated risk factors such as BMI, insulin resistance, alcohol ingestion, or use of medications known to cause steatosis. Consistently, Hispanics were reported to have a disproportionately high prevalence of NAFLD-related cirrhosis while that of Blacks was disproportionately low[21]. Studies from children and adolescents have generated similar findings in regards to ethnic disparities in the prevalence of NAFLD[22]. Mexican-American children tend to have a higher rate of fatty liver even after controlling for the severity of obesity. In contrast, African-American children have a relatively lower prevalence despite their increased risk factors such as obesity and insulin resistance.

Genomic aspect

Recently, the importance of genetic variation in etiology of NAFLD has been increasingly recognized. Accumulated numbers of single nucleotide polymorphisms

(SNPs) associated with NAFLD have been documented by candidate gene studies. Many variations in candidate genes could contribute to the pathogenesis of NAFLD, including genes related to insulin resistance pathway, genes impacting hepatic lipid metabolism, cytokine-related genes, and genes encoding endotoxin receptors[23]. Among these genes, peroxisome proliferatoractivated receptor-γ (PPAR-γ), adiponectin, leptin, and tumor necrosis factor-α (TNF-α) were frequently reported[24]. With the novel application of genome-wide association studies (GWASs), there have been significant advances in our understanding of genomic variations of NAFLD. Patatin-like phospholipase domain containing family member A3 (PNPLA3) has caught most attention. An allele of PNPLA3 (rs738409, encoding I148M) on chromosome 22, most commonly seen in Hispanics, has been shown to consistently associated with increased hepatic fat and hepatic inflammation by a series of studies[25-29] although the physiological role of PNPLA3 in NAFLD remains unknown.

In addition, the investigation into epigenetic modification in NAFLD etiology is rapidly growing and most extensively focused on microRNA. MicroRNAs (miRNAs), typically 22 nucleotides in length, are non-protein-coding, small single-stranded RNA and regulate gene expression via messenger RNA degradation or translational inhibition[30]. One of the best-characterized miRNAs in NAFLD is the hepatocyte-specific miRNA-122, which accounts for ~70% of miRNAs found in the liver. Studies in mice suggested that chronic repression of miRNA-122 could develop hepatosteatosis possibly as a consequence of global impairment of lipid metabolism[31]. Consistently, this finding was supported and further extended in a clinical study that miRNA-122 was found to be significantly under-

expressed in subjects with NASH compared to controls with normal liver histology[32]. Some other miRNAs such as miRNA-34a and miRNA-146b were also shown to be altered in NAFLD patients[32]. However, the genomic and epigenetic knowledge related to NAFLD are novel and still need to be validated and refined by larger replications.

Diet and lifestyle

It is believed that diet and other lifestyle practices have a strong influence on the development and progression of NAFLD. Musso et al. analyzed the diet of 25 patients with histologically confirmed NASH and suggested that development of NASH was associated with a diet rich in saturated fat and poor in polyunsaturated fat and fiber[33]. Deficient levels in antioxidants (eg, vitamin C and E) were also reported in this study and confirmed by others[34]. Responsible risk factors for NAFLD might also include choline deficiency[35], high fructose consumption (primarily from soft drinks)[36], and lack of exercise[37]. In Chapter II.2, we will carefully review and discuss the significance of fructose consumption in NAFLD, as well as its hepatic metabolism and adverse health outcomes.

1.5 Therapeutics

NAFLD is frequently associated with obesity, and obesity can lead to the occurrence of other metabolic abnormalities such as dyslipidemia and insulin resistance, which are risk factors for NAFLD. Weight loss is usually recommended as first-line treatment for

NAFLD patients. This can be achieved through multifaceted lifestyle interventions including diet restriction, physical exercise, and behavioral counselling; and through weigh-reducing drugs and surgery for advanced disease. In a clinical trial, overweight and obese healthy adults submitted to either reduced carbohydrate or reduced fat hypocaloric diets showed a significant improvement in weight, body fat, and intrahepatic triglycerides content over 6 months[38]. Consistently, several well-structured programs of weight loss were associated with improvement in liver enzymes, insulin sensitivity, and hepatic fat[39,40]. However, these positive findings are limited to relatively short term studies and could be affected by lack of adherence over a longer time period.

Treatment of NAFLD also includes insulin-sensitizing drugs (eg, Metformin, Pioglitazone). Other commonly prescribed medicines include lipid-lowering drugs (eg, Probucol, Atorvastatin) and cytoprotective agents (eg, anti-oxidants). Targher et al. pointed out that pharmacotherapy for NAFLD should be carefully reserved for patients with NASH who are at the highest risk of disease progression[41], probably because of detrimental effects of several drugs. For instance, the insulin sensitizer, rosiglitazone, was demonstrated to improve hepatic steatosis and features of hepatic inflammation and fibrosis, but it was associated with a significant increase in the risk of myocardial infraction and of death from cardiovascular causes[42,43]. Some lipid-lowering agents are also concerning for their safety due to the presentation of liver toxicity[44]. Therefore, there is still a lack of consensus for definitive recommendations regarding the appropriate pharmacological therapy for NAFLD.

Taken together, no highly effective options are currently available for NAFLD treatment, thus interventions such as emphasizing on modifiable dietary factors should be explored and evaluated to prevent disease progression and to manage NAFLD-associated complications.

1.6 Associated risk - cardiovascular disease

NAFLD in both adults and children is frequently reported to be associated with metabolic syndrome features[45], including abdominal obesity, hypertension, insulin resistance, as well as atherogenic dyslipidemia (typically high triglyceride and low HDL cholesterol phenotype in NAFLD). The presence of metabolic syndrome and its individual components increase the likelihood of having cardiovascular disease (CVD)[46]. Recent studies have clearly documented a strong association between NAFLD and atherosclerosis. Adults with biopsy-proven NAFLD were demonstrated to have greater carotid intima media thickness (cIMT), a reliable index of subclinical atherosclerosis, when compared with matched healthy controls; and this increase of cIMT was significantly associated with the histological severity of NAFLD[47,48]. Endothelial flow-mediated vasodilation, another marker of subclinical atherosclerosis, was also suggested to be impaired in NAFLD patients[49]. Furthermore, an expanding body of evidence supports the increased prevalence of clinical cardiovascular events in adults with NAFLD, even after adjusting for demographic and metabolic factors[50,51]. These findings obviously raise the question as to whether CVD events will occur earlier than end-stage liver disorders and liver failure. Therefore, several natural history studies in adults have been

conducted to evaluate long-term morbidity and mortality rate in NAFLD. In particular, Rafiq et al. examined 173 individuals with biopsy-proven NAFLD and suggested coronary artery disease was the leading cause contributing to the increased mortality rate, exceeding the risk from malignancy and liver-related mortality[52]. In a community-based cohort of 420 NAFLD patients, Adams et al. consistently reported that total mortality was increased during a mean follow-up of 7.6 years in NAFLD patients compared with general population, and was most commonly because of ischemic heart disease and malignancy[53]. In the pediatric population, although they have not been followed long enough to examine whether they have increased cardiovascular events in their later life, many adolescents with NAFLD already manifest early signs of subclinical atherosclerosis[54]. This is particularly worrisome because studies from adults have shown that substantial mortality is caused by cardiovascular-related events and patients with NAFLD probably die early of CVD prior to liver failure.

The underlying biological mechanisms leading to CVD in patients with NAFLD remain unknown. It is possible that NAFLD, especially in its more advanced forms, acts as a stimulus for further increased whole body insulin resistance and dyslipidemia, contributing to accelerated atherosclerosis. Another possible mechanism linking NAFLD to CVD could be represented by the systemic release of several inflammatory and oxidative-stress mediators, which are thought to be detrimental to vessels. Finally, NAFLD might play a role in the pathogenesis of atherosclerosis through some coagulant factors such as plasminogen activator inhibitor-1 (PAI-1)[41,55].

II.2 Dietary Fructose

2.1 Trend of consumption in general population

Fructose can be found in its monosaccharide form or can be bound to glucose with a disaccharide bond in sucrose. The primary dietary sources of fructose are from high fructose corn syrup (typically 55% free fructose and 45% glucose) and sucrose (a disaccharide composing equally of fructose and glucose) which are widely used to sweeten beverages and many processed foods. According to the NHANES III data, average fructose consumption was estimated to have a ~50% increase from 1977-1978 (37 g/day, 8% of total caloric intake) to 1988-1994 (54.7 g/day, 10.2% of total caloric intake)[56]. The most important contributor was sugar-sweetened beverages (SSB) that account for 30% of total fructose consumption. In particular, the contribution from SSB was the highest in adolescents, representing approximately 45%[56]. As further analyzed by Wang et al., per-capita daily caloric contribution from SSB in U.S. youth aged 2-19 years increased to 224 kcal in 1999-2004 as compared to 204 kcal in 1988-1994[57]. In 2005, the American Heart Association published dietary guidelines for children and adolescents and recommended that "sweetened beverages and naturally sweet beverages, such as fruit juice, should be limited to 4 to 6 oz per day for children 1 to 6 years old, and to 8 to 12 oz per day for children 7 to 18 years old"[58]. Recently, possibly in part due to the increased public awareness of the negative health consequences of excessive sugar, total consumption of added sugars including SSB has slightly reduced in adults[59]; however, the overall intake by many Americans is still believed to exceed dietary recommendations.

2.2 Increased consumption in patients with NAFLD

Several lines of evidence suggest an association between fructose consumption and NAFLD. A cross-sectional study examined 349 volunteers and reported that those with NAFLD identified by abdominal ultrasound consumed almost twice amount of soft drinks comparing to participants with normal liver[60]. Consistently, a small case-control study in patients with biopsy-proven NAFLD also demonstrated a higher fructose intake from sweetened beverages than their age, gender, and BMI matched controls[61]. Furthermore, Abdelmalek et al. evaluated histological features of a large cohort of adults in NAFLD and found that increased ingestion of fructose significantly correlated with the severity of fibrosis[62].

2.3 Hepatic metabolism

Dietary fructose is absorbed into the intestine via a saturable, facilitative transporter GLUT5. After absorption across the brush boarder of the small intestine into the portal blood supply, fructose is primarily metabolized in hepatocytes in a relatively unregulated fashion. Distinct from glucose, fructose can bypass the rate-limiting step of phosphofructokinase and is rapidly phosphorylated to form fructose-1-phosphate in a reaction catalyzed by fructokinase. Accumulated fructose-1-phosphate could further result in the production of glyceraldehyde, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate, which are substrates for the synthesis of fatty acids and consequently promoting hepatic fat deposition.

2.4 Fructose-induced negative health outcomes

Hypertriglyceridemia

Feeding studies in adults have consistently reported that fructose, in various exposure durations and doses, induces increased plasma triglycerides at both fasting and postprandial states. With a cross-over study design, Teff et al. demonstrated that high fructose (30% of total calories) diets produced a rapid and prolonged (24 hours) elevation of plasma triglycerides in young, healthy females[63]. This finding was further supported by studies with longer feeding periods that last 6 days, 2 weeks, 3 weeks, 4 weeks, and 10 weeks[64-67]. The possible mechanism responsible for this fructose-induced hypertriglyceridemia appears to be increased *de novo* lipogenesis (DNL) because fructose is demonstrated to activate sterol receptor element binding protein-1c (SREBP-1c) independently of insulin, which then activates genes involved in DNL[68]. Greater rates of DNL can promote increased hepatic very low density lipoprotein (VLDL) secretion and a resultant delay of clearance by lipoprotein lipase (LPL). Decreased LPL activity was also found after consuming fructose as compared to glucose[69]. Together, increased concentration of larger VLDL, in combination with longer VLDL residence time, would result in hypertriglyceridemia and concomitantly allow for augmented lipoprotein remodeling to generate small, dense LDL particles, thereby creating a pro-atherogenic environment.

Visceral adiposity, inflammation, and insulin resistance

As compared with the sugar glucose, fructose induces lower postprandial glucose and insulin excursions. Paradoxically, chronic exposure to high fructose causes increased plasma insulin concentration and insulin resistance. Much evidence has suggested the close relationship between fructose consumption and insulin resistance[67,70] although the underlying mechanism(s) are not fully understood. Stanhope et al. proposed that it might be due to the increased triglyceride uptake by visceral adipose tissue[69]. Fructose triggers lower post-meal insulin peak that could lead to reduced insulin-mediated LPL activity in subcutaneous adipose tissue, which allow for the preferential deposition of fat in the visceral adipose tissue (VAT). Indeed, greater consumption of fructose has been suggested to be associated with the increased visceral adiposity in both adults and children[67,71]. Expanded VAT is metabolically active and can produce numerous inflammatory cytokines such as TNF-α, interleukin-6 (IL-6), and C-reactive protein (CRP). These inflammatory cytokines could mediate several signaling pathways leading to insulin resistance, including the nuclear factor κB (NF-κB) and the c-Jun N-terminal kinase (JNK) pathways.

Oxidative Stress

In animal model studies, fructose has been reported to promote oxidative damage by reducing antioxidant defenses and enhancing the production of reactive oxygen species (ROS)[72-75]. Recently, this fructose-induced oxidative stress was demonstrated in healthy adult volunteers, indicated by the increases in urinary F2-isoprostanes, thiobarbituric

acid–reactive substances (TBARS) and protein carbonylation in muscular tissues[76]. Oxidative stress has been proposed as a critical "second hit" driving the progression from simply steatosis to NASH, and as an important mediator involved in the pathogenesis of atherosclerotic lesions. Consumption of high fructose provoked superoxide production in the aortas and decreased vasoprotective factors in rats[77]. However, evidence has not been well documented in human trials and need to be carefully examined in the future.

Endotoxemia

Endotoxin, which is found in the outer cell membrane of gram-negative bacteria, can be absorbed from gastrointestinal tract into the blood circulation via translocation. Bergheim et al. reported that moderate fructose consumption lead to increased intestinal translocation of bacterial endotoxin, and subsequently liver steatosis in mice[78]; and their findings were further extended in non-human primates, a more relevant animal model to human[79]. Although fructose-induced endotoxin effects are less well understood in humans, evidence from animal studies have proposed that fructose ingestion is associated with marked loss of tight junction protein and intestinal bacterial dysbiosis, resulting in increased circulating endotoxin[80]. Elevated endotoxin levels can activate toll like receptor 4 (TLR4) and trigger the downstream inflammatory signaling cascade[81,82], thereby promoting insulin resistance and liver injury[83,84].

II.3 Purpose of Research

Given the intertwined relationship between NAFLD and CVD, underlying atherosclerotic risk factors in NAFLD need to be aggressively managed, as many subjects with NAFLD will have major morbidity and mortality from cardiovascular events. Exploring early prevention through modifiable dietary factors is a feasible, side-effect free strategy. Because fructose-induced dyslipidemia, insulin resistance, and oxidative damage could specifically contribute to the increased CVD risk seen in NAFLD, a comprehensive dissertation research plan was well designed to evaluate fructose consumption and cardiovascular risk in NAFLD. In particular, this research plan has a focus on U.S. adolescents in view of the fact that adolescents have continuously increased prevalence of NAFLD[12] and the highest intake of fructose (primarily from sweetened-beverages)[56]. The specific research aims included:

1) To determine whether fructose consumption could acutely alter/exacerbate the lipid profile, glycemic status, and other CVD risk factors in adolescents with NAFLD;

2) To determine if fructose restriction could improve hepatic steatosis, lipids, insulin resistance, and other markers related to CVD risk in adolescents with NAFLD; and

3) To examine whether endotoxin could be a possible mediator through which fructose induces CVD risk as seen in adolescents with NAFLD.

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Chapter III. Children with NAFLD Are More Sensitive to the Adverse Metabolic Effects of Fructose Beverages than Children without NAFLD

Jin R, Le NA, Liu S, Farkas Epperson M, Ziegler TR, Welsh JA, Jones DP, McClain CJ, Vos MB. Children with NAFLD are more sensitive to the adverse metabolic effects of fructose beverages than children without NAFLD. *J Clin Endocrinol Metab*. 2012 Jul; 97(7):E1088-98.

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Abbreviated Title: Effects of Fructose in Children

Key Terms: Added sugars, Adolescents, Cardiovascular disease, Children, Dyslipidemia, Fatty Liver, Fructose, HDL, NAFLD, Non-esterified fatty acids, Pediatric NAFLD, Triglycerides

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Abstract

Context: Dietary fructose induces unfavorable lipid alterations in animal models and adult studies. Little is known regarding metabolic tolerance of dietary fructose in children.

Objectives: To evaluate whether dietary fructose alters plasma lipids in children with nonalcoholic fatty liver disease (NAFLD) and in healthy children.

Design and Setting: We performed a 2-day, cross-over feeding study at the Inpatient Clinical Interaction Site of the Atlanta CTSI at Emory University Hospital.

Participants and Intervention: Nine children with NAFLD and ten matched controls without NAFLD completed the study. We assessed plasma lipid levels over 2 non-consecutive, randomly assigned, 24-hour periods under isocaloric, isonitrogenous conditions with three macronutrient-balanced, consecutive meals and either 1) a fructose-sweetened beverage (FB) or 2) a glucose beverage (GB) being consumed with each meal.

Main Outcome Measures: Differences in plasma glucose, insulin, triglyceride (TG), apolipoprotien B (apo B), high density lipoprotein cholesterol (HDLc) and non-esterified free fatty acid (NEFA) levels were assessed using mixed models and 24-hour incremental areas under the time-concentration curve (IAUC).

Results: After FB, TG IAUC was higher versus after GB in both children with NAFLD ($p=0.011$) and those without NAFLD ($p=0.027$); however, incremental response to FB was greater in children with NAFLD versus without NAFLD ($p=0.019$). For all subjects, HDLc declined in the postprandial and overnight hours with FB but not with GB ($p=0.0006$). NEFA were not impacted by sugar but were significantly higher in NAFLD.

Conclusions: The dyslipidemic effect of dietary fructose occurred in both healthy children and those with NAFLD; however, children with NAFLD demonstrated increased sensitivity to the impact of dietary fructose.

Introduction

Pediatric nonalcoholic fatty liver disease (NAFLD) is a chronic, obesity-associated liver disease that is the most common liver disease in children today. It is estimated to affect over 6 million children in the United States[1], and because of the obesity epidemic, its prevalence is increasing. NAFLD can result in end stage liver disease[2], but it is also associated with an increase in cardiovascular disease (CVD) and all-cause mortality[3-5].

Children with NAFLD have increased fasting triglycerides[6-9] and lower high-density lipoprotein cholesterol (HDLc)[8-10], a pattern strongly associated with increased CVD risk[11] and typical of insulin resistance syndromes. The presence of dyslipidemia in adolescents and children is particularly worrisome because dyslipidemia in youth predicts increased CVD risk in adulthood[12]. While children with NAFLD have not yet been followed long enough to know if the dyslipidemia predicts increased cardiovascular events later in life, adults with NAFLD have increased mortality from cardiovascular events even after adjusting the presence of metabolic syndrome[13]. This suggests that a component of NAFLD increases atherosclerosis, independent of other known risk factors including obesity and central adiposity. Similar to other insulin resistant states, patients with NAFLD typically have the combined pattern of dyslipidemia characterized by fasting and post-prandial hypertriglyceridemia, low HDL as well as smaller HDL and LDL particles and accumulation of VLDL[14].

Fructose is a common nutrient in the diets of children (representing approximately 11% of calories for a typical U.S. adolescent[15]) in the US and it has previously been reported

to be associated with NAFLD severity in adults[16-18]. Unlike glucose, fructose is metabolized in the liver in a first-pass process without being involved in the rate-limiting phosphofructokinase pathway. High fructose consumption has been shown to increase hepatic de novo lipogenesis within hours[19] and contributes to increased hepatic TG synthesis first via increased glycerol synthesis and second through increased formation of saturated fatty acids, primarily palmitate[20]. In animal studies, a high fructose diet increases de novo lipogenesis, adiposity, plasma triglycerides, hepatic fat content and oxidative stress[21-24]. Short term feeding studies in adults indicate higher plasma triglyceride levels, both fasting and post-prandial when fructose is substituted for glucose.[20,25-29]

In spite of the strong role of fructose consumption in hepatic steatosis and CVD risk as well as the high consumption of fructose-containing beverages among pediatric population, studies on the effect of fructose in children are lacking. Improving our understanding of dietary contributors to cardiovascular risk could lead to a potential long term prevention strategy of CVD in pediatric NAFLD. We hypothesized that in children with NAFLD, fructose beverages would exacerbate dyslipidemia seen in the post prandial and overnight time periods and that this effect would not be seen in healthy weight, metabolically normal children. We undertook a 24-hour feeding study of children with NAFLD and in matched overweight and normal healthy (non-NAFLD) weight controls comparing fructose beverages to glucose beverages to assess this hypothesis.

Subjects and Methods

Study Design: This was a randomized, double-blind, cross-over 2-day feeding study. Two separate visits were designed to compare responses to meals with glucose-containing beverages (GB) and to meals with isocaloric fructose-containing beverages (FB). This study was approved by the Emory and Children's Healthcare of Atlanta IRB and consent and assent (when applicable) were obtained for each subject prior to initiation of the study. The study was conducted at Emory University Hospital Clinical Interaction Network site of the Atlanta Clinical and Translational Science Institute.

Subjects: A total of 20 children (age range 11 to 18 years) met eligibility criteria and attended both visits of the study. Subjects with NAFLD (confirmed by liver biopsy within the past 2 years) were recruited from Emory Children's Center Liver Clinic; and non-NAFLD controls (defined as hepatic fat infiltration < 3% by MRS) were recruited at the Emory Children's Center general pediatric clinics. Of the subjects with NAFLD, on liver biopsy, 1 subject had simple steatosis, 7 had non-alcoholic steatohepatitis (NASH) and 2 had NASH with bridging fibrosis. Controls were matched by age, gender and race/ethnicity to the children with NAFLD. Healthy controls were recruited regardless of weight and during screening were characterized as either normal weight (BMI < 85^{th} percentile) or overweight/obese (BMI $\geq$ 85^{th} percentile). Inclusion criteria included that they were healthy (without chronic illness including diabetes or need for daily medications), normal ALT/AST, and negative MRI for hepatic steatosis. Exclusion criteria for both groups included fever within the past 2 weeks, pregnancy, unable to

obtain an MRI and renal insufficiency. One subject with NAFLD was excluded from the analysis due to incomplete blood collection.

Study Methods: For each of two visits, subjects consumed a standardized meal consisting of 700 kcal and 78 grams of sugar the evening prior to the study and then fasted for 12 hours prior to the initial baseline measurements. Subjects arrived at the inpatient research unit at 0700 and had an IV placed for blood draws. Blood samples were drawn at 0800 (baseline), 1 hour after breakfast and subsequently, every 2 hours until the following morning except at 0400 to allow the children to sleep. Standardized meals (50% carbohydrates, 30% fat and 20% protein) were prepared in the metabolic kitchen of the research unit by the research nutritionist, with 33% of total estimated daily calories (1.3 x estimated needs using calculated basal metabolic rate using the Harris Benedict formula) provided as an isocaloric, sugar-sweetened beverage containing either glucose or fructose. Meals were typical for children and included items such as scrambled eggs, a hamburger, tater tots, and green beans. The children were randomized to either glucose (as dextrose) beverages or fructose (granulated fructose dissolved in water) beverages at Visit 1 and the other sugar at Visit 2. For each subject, lunch was served 4 hours after breakfast and dinner was served 8 hours after breakfast. Typical meal times for a subject were 0800 h (breakfast), 1200 h (lunch) and 1600 h (dinner), after which only ad lib water was allowed. A washout period of 5-14 days was included between the two study visits. Participants were instructed not to change dietary habits during the washout period.

Imaging: Each child underwent a MRI/MRS to evaluation for hepatic lipid volume, typically during the first study visit. The hepatic lipid percentage of participants was calculated by magnetic resonance spectroscopy (MRS) using our previously described methods[30,31]. In brief, after acquisition, water and lipid magnitude spectra were analyzed by determining the area under the curve corresponding to a user-defined frequency range surrounding the corresponding water/lipid peaks (water peak: 4.6ppm; lipid peak: 1.3, 2.0ppm). The integrated magnitude signals at each TE were fit to exponential T2 decay curves, whereby the equilibrium signal (M_0) and the relaxation rate (R2=1/T2) were determined by least-squares approximation. Using M_0 for water and lipid, the T2-corrected hepatic lipid fraction was calculated from: %Hepatic Lipid = M_0lipid / (M_0lipid + M_0water).

Lipids & Clinical measurements: Blood samples were collected in EDTA tubes, protected from light, and transported on ice to the laboratory for immediate processing. The extra samples were stored at -80°C. All lipid measurements were performed by the Emory Lipid Research Laboratory, and lab specialists were blinded to all subject information during sample processing and analysis. Total cholesterol, triglycerides and glucose were determined by enzymatic methods using reagents from Beckman (Beckman Diagnostics, Fullerton, CA). LDL-cholesterol (LDLc) and HDL-cholesterol (HDLc) were determined using homogeneous enzymatic assays (Sekisui Diagnostics, Exton, PA). Non-esterified fatty acids (NEFA) were measured by colorimetric methods using reagents from (Wako Chemicals). Apolipoproteins (apo), insulin and high sensitivity C-reactive protein (hs-CRP) were measured using immunoturbidometric methods (Sekisui

Diagnostics, Exton, PA). ALT and AST were performed by the clinical laboratory at Emory University Hospital.

Statistical Analyses: Sample size calculation was determined based on expected difference in triglyceride area under the curve in the NAFLD group using previously reported data (28). We estimated that N=6 would be necessary to attain a power of 0.80 to identify a true difference in TG level in response to fructose compared to glucose. Additional subjects were enrolled because the response to TG was expected to be lower in children and to allow for potential variability caused by NAFLD. Results were expressed as mean (SE). Independent two sample t-tests were used for comparison between non-NAFLD and NAFLD groups. Paired sample t-tests were conducted for comparison between two separate visits, and p-values were considered as significance when < 0.05. Missing data imputation methods were used for the small number of missing data points (<1%). Due to the lack of linear pattern with the time points, if a patient had a missing value at a certain time point, the average change plus the average mean of the outcome at its adjacent time points was used to replace the missing data. For variables of plasma glucose, insulin, TG, apoB, TG/apoB ratio, comparisons of 24h response were made using the incremental area under the curve (IAUC) using the trapezoidal method and paired analysis comparing individual time-points at baseline (0 hour, fasting), post-prandially (9 hours, post meal assessment) and the post-test fasting (23 hours, fasting). Since the response variables were measured at multiple time-points throughout the 24-hr period, the linear mixed model analysis was utilized to compare the effects of the two different beverages as well as the influence of NAFLD disease.

Specifically, a random intercept model was assumed for each individual to incorporate the within-subject correlation and those random effects represent the influence of the repeated observations from the same subject. HOMA-IR was calculated from insulin and glucose at fasting state [glucose(mg/dL)*insulin(μU/mL)/405] and entered into the mixed model to test the effect of insulin resistance on response to sugars.

Results

Clinical Characteristics of the Study Population

Clinical characteristics of the two groups are described in **Table 1**. Non-NAFLD controls were matched to NAFLD subjects by age, gender, and race. In the control group, 5 subjects had a BMI $\geq 85^{th}$ percentile for weight and gender while 5 subjects were between the 5^{th} and 85^{th}percentile. At baseline, subjects with NAFLD had increased BMI z-score ($p=0.003$), hepatic lipid ($p<0.001$), ALT ($p<0.001$), AST ($p<0.001$), total cholesterol ($p=0.022$), apoB ($p=0.034$), insulin ($p=0.001$), HOMA-IR ($p=0.015$), and hs-CRP ($P=0.008$), compared to the non-NAFLD controls (Table 1). Although there was some intra-subject variability in the fasting levels, baseline clinical measurements were not significantly different between the two visits for subjects.

Effect on Plasma Glucose and Plasma Insulin

There was no difference in fasting plasma glucose between the two groups; however NAFLD subjects had significantly higher insulin levels and higher HOMA-IR (Table 1),

indicative of insulin resistance. The acute increases in glucose (**Figure 1A-1B**) and insulin (**Figure 1C-1D**) were greater after consumption of GB as compared to FB for both NAFLD (p=0.067) and non NAFLD (p=0.095) subjects. NAFLD patients exhibited a greater net increase, but the differences did not reach statistical significance possibly due to the attenuated effect after dinner. At the end of the FB visit (time point of 23h), fasting plasma glucose remained significantly higher as compared to that after one day GB feeding, for both NAFLD (p=0.046) and non-NAFLD (p=0.008) (**Figure 1A-1B**). NAFLD participants had higher postprandial insulin response (24h-IAUC) as compared to non-NAFLD controls during both the FB (mean ± SE: 996.3 ± 189.8 vs. 451.6 ± 228.6, p=0.028) and GB periods (mean ± SE: 1444.9±247.8 vs. 591.4±239.9, p=0.008). In non-NAFLD controls, postprandial insulin response (24h-IAUC) was significantly increased after GB as compared to FB (mean ± SE: 591.4 ± 239.9 vs. 451.6 ± 228.6, p=0.021) (Figure 1C-1D). There was no difference in insulin IAUC between the two periods for NAFLD subjects.

Effect on Plasma Non-Esterified Fatty Acids

Fasting NEFA levels were not significantly different between the two groups (Table 1). In the non-NAFLD controls, NEFA levels decreased as expected after the breakfast-induced increase in plasma insulin and remained suppressed throughout the daytime hours followed by a rise overnight during fasting (**Figure 2A**). In NAFLD children, highly abnormal NEFA patterns were seen including a lack of the expected sustained post-prandial decrease (**Figure 2B**) and overall increased levels compared to the healthy

children (mixed model, beverage: p = 0.260; disease: p = 0.002; beverage x disease interaction: p = 0.558). No differences were seen between the FB and GB periods.

Effect on Plasma Triglycerides

Children with NAFLD had higher fasting triglyceride levels (**Table 1**) and had higher 24-hr postprandial triglyceride response during both FB (mean±SE: 1421.2±267.1 vs. 622.4±110.2, p=0.019) and GB (mean±SE: 757.3±136.5 vs. 325.7±87.6, p=0.015) periods as compared to subjects without NAFLD; and the 24-hr IAUC of plasma TG was significantly increased by consumption of FB in contrast to GB, for both children with NAFLD (mean±SE: 1421.2±267.1 vs. 757.3±136.5, p=0.011) and children without NAFLD (mean±SE: 622.4±110.2 vs. 325.7±87.6, p=0.027), respectively (**Figure 3A-3B**). The 24-hr IAUC was not correlated with the baseline plasma TG concentration. The results of the 24-hr IAUC calculation concurred with the result from mixed model (beverage: p<0.0001; disease: p=0.008; beverage x disease interaction: p=0.396). When including baseline HOMA-IR into the mixed model, TG response was also affected by insulin resistance (beverage: p<0.0001; disease: p=0.050; HOMA-IR: p<0.0001; beverage x disease interaction: p=0.890). At the end of visit (time point of 23h), fasting plasma TG was significantly elevated after FB compared with GB for both NAFLD (p=0.011) and non-NAFLD (p=0.005) subjects (Figure 3A-3B).

Effect on Plasma apoB

NAFLD subjects had higher plasma apoB at baseline as compared to non-NAFLD due in part to higher plasma triglycerides and a slight elevation in LDLc (**Table 1**). Over the two 24-hr metabolic studies, there was minimal change in plasma apoB in either group (**Figure 3C-3D**). In view of the fact that there was no change in LDLc during the two periods with either group (data not shown), the postprandial TG/apoB response is assumed to indirectly reflect the change in composition of the triglyceride-rich lipoproteins. Compared to GB, FB consumption was associated with greater postprandial response in TG/apoB, for both NAFLD ($p<0.0001$) and non-NAFLD ($p=0.030$) groups (**Figure 3E and 3F**), and NAFLD exacerbated the effect of fructose ($p=0.018$), but not glucose, on TG/apoB (mixed model, beverage: $p<0.0001$; disease: $p=0.045$; beverage x disease interaction: $p=0.025$).

Effect on HDLc

HDL-C levels were similar at baseline in NAFLD patients compared to non-NAFLD controls (Table 1). In both groups, HDLc was significantly lower after consuming FB as compared to GB (**Figure 4A-4B**). No difference in HDL response was found between NAFLD and non-NAFLD subjects (mixed model, beverage: $p=0.0006$; disease: $p=0.478$; beverage x disease interaction: $p=0.382$).

Discussion

Concurring with studies performed in adults[20,27,28,32], we found that fructose exacerbates post prandial lipemia in children with and without NAFLD. Based on these preliminary,

yet novel data, we have reached the following conclusions: (a) in the setting of nutrient balanced meals, fructose beverages induce post-prandial and overnight increases in plasma triglyceride levels in children with and without NAFLD that does not return to baseline fasting levels by the following morning, (b) in NAFLD subjects, the marked increase in TG appears to be from an increase in size of TG-rich lipoprotein particles, as demonstrated by an increased in TG-to-apoB ratio (c) fructose beverages result in a rapid decrease of HDL-c post-prandially and overnight supporting the hypothesis that trigylceridemia results in accelerated catabolism of HDL in both children with NAFLD and who are healthy and (d) NEFA levels were not different after FB vs GB but did appear highly abnormal in the children with NAFLD as demonstrated by a lack of post-prandial suppression of NEFA.

Hypertriglyceridemia

The effect of carbohydrates in raising plasma triglycerides is well known, although the relative contribution of the different sugars has been debated. As early as 1961, Ahrens *et al.* reported a fasting lipemia after a fat-free, high carbohydrate diet[33]. This lipemia was demonstrated by others to consist of an increase in large-TG rich VLDL and alterations in the catabolism of VLDL explained the prolonged hypertriglyceridemia following a high-carbohydrate challenge[34,35]. Our findings are similar to the response to fructose documented by Teff *et al.*, who studied healthy women given sugar beverages with meals over a 24-hour period[27]. They demonstrated that plasma triglycerides were significantly elevated after fructose compared to glucose several hours after each meal and this effect persisted even 12 hours after the fructose beverage[27]. More recently,

investigators have focused on fructose specifically and, in adults, found that it increases plasma TG in studies lasting 1 day[27],2 weeks[32], 6 days[36], 4 weeks[37] and 12 weeks[38] when compared to glucose feeding. Population data in adults demonstrate increased plasma TG and lower HDL in association with increased added sugar consumption, suggesting that the "high carbohydrate effect" is sustained over time[39].

In our subjects with NAFLD, fructose increased post prandial TG and resulted in a significantly increased IAUC for TG for the 24 hours. This increase may result in part from the fact that dietary fructose is less tightly regulated than glucose, bypassing the rate-limiting step in glycolysis of phosphofructokinase, resulting in increased production of acyl-CoA, a precursor in *de novo* lipogenesis. While we were unable to measure VLDL-TG size directly in our study given volume limitations in children, Chong *et al.* demonstrated that the hypertriglyceridemia after fructose is associated with a significant increase in VLDL-TG particle size and a delay in clearance in VLDL-TG, likely from competitive inhibition[20]. Tracer kinetic studies have indicated an increase in TG production with no change in apoB production, a further demonstration of increased VLDL particle size[35]. The increase in TG-to-apoB ratio in our subjects indirectly supports these findings, although data on the TG-to-apoB ratio in isolated VLDL would have been more conclusive. In NAFLD, VLDL-TG from *de novo* synthesis is 4-5 times higher than expected compared to healthy individuals, and fails to vary with fasted and fed state[40]. Without concomitant increase in VLDL apoB production, the size of secreted VLDL would be expected to be increased.

HDL

Clinical observation has demonstrated that there is a close relationship between hypertriglyceridemia and reduced HDL levels. Significant inverse correlations have been documented between these two lipid parameters[41,42] and high triglycerides and low HDL-c is the typical dyslipidemia that is closely associated with insulin resistance syndromes[43], including NAFLD. TG elevation is hypothesized to affect HDL in at least two important ways 1) decrease hydrolysis of triglyceride rich lipoproteins (TRL) would reduce materials available as precursors for plasma HDL and 2) increased size and number of TRL could enhance the transfer of TG from TRL to HDL by cholesteryl ester transfer protein (CETP), resulting in HDL that are relatively TG enriched and CE depleted[42]. Studies directly testing these hypotheses have demonstrated increased fractional catabolic rate of apoA-I but not a reduction in apoA-I production rates[42], supporting the hypothesis of enhanced clearance of HDL particles. Rashid *et al* tested this and demonstrated that a 3-6% increase in TG content of HDL resulted in a 26% increase in the fractional catabolic rate of HDL apoA-1[44]. In our subjects, fructose beverages were followed by prolonged elevation of plasma TG and an acute decrease in HDLc levels. The acute timing of the change supports accelerated catabolism as opposed to alterations in HDL production. Although the change in HDLc was slightly less robust in the healthy subjects compared to NAFLD subjects, both groups had a significant decreases in HDLc. Others have examined HDL after longer term studies of fructose and the results have been variable. Bantle *et al* studied a high fructose diet (17% of energy) in healthy adults and did not find a significant decrease in HDL, although their data demonstrated a trend down from 54 mg/dL to 50 mg/dL with continued administration of fructose[25]. We

previously evaluated cross sectional population data and found significantly reduced HDL levels in both adults and adolescents who consumed higher amount of added sugar (the largest source of fructose), suggestive of a chronic effect[39,45]. Interestingly, in our cross sectional study, BMI did not modify the added sugar and HDL relationship. Similarly, in this current study, the acute changes in HDL were not affected by BMI or level of insulin resistance.

NEFA

A surprising finding in our study was the significant increase in daylong levels of NEFA in NAFLD compared to non-NAFLD, despite similar fasting values at baseline. Elevated NEFA levels have been associated with atherogenesis[46] and increased plasma NEFA may contribute to hepatic steatosis through increased esterification of fatty acids flowing to the liver resulting in increased TG. In the healthy fasting state, plasma NEFA provides the majority of the fatty acids used to synthesize hepatic derived VLDL-TG[47]. After feeding, the rate of NEFA returns from adipose tissue should decline and total plasma level should be decreased as seen in our healthy controls. Although neither group demonstrated a differential response to fructose compared to glucose, supporting previous findings by Parks et al[19], in the NAFLD subjects, we observed a shortening of the post-prandial nadir for NEFA after breakfast and NEFA was increased throughout the day. This is markedly different from the healthy, expected pattern. Free fatty acid dynamics may be critical to the mechanism(s) of NAFLD because increased plasma NEFA may increase the load of fatty acids on the liver and be a stimulus for the increased rate of re-esterification seen in NAFLD[19]. In addition, increased NEFA have been shown

to cause hepatic lipotoxicity[48,49] and have been associated with increased severity of NASH in adult patients[50]. Our data confirm that NEFA flux is highly dysregulated in children with NAFLD compared to healthy controls.

Strengths and Limitations

Overall, our study supports the need for longer term studies of fructose reduction in children with NALFD in particular. One limitation of our study is the short term nature of the results. Some studies, including animal models suggest that there may be adaption to longer term high carbohydrate diets that would ameliorate the dyslipidemic effects. While it is difficult to study diet effects in children in longer term studies because of subject diet variability, we previously evaluated cross sectional data representative of the US and found a strong association between added sugar consumption (the primary source of fructose) and TG and HDL[39,45]. Specifically, adolescents who consumed diets high in added sugars had increased TG and lower HDL levels[45]. Others have shown in a cross sectional study of adolescents that fructose consumption was associated with increased visceral adiposity which then predicted increase CVD risk[51]. Together, these two studies suggest that the short-term alterations we demonstrated in children likely have sustained effects as well.

We were unable to assess the effect of fructose directly on the liver in the children with NAFLD. In our clinical experience decreasing soda intake (the largest source of fructose in a typical child/adolescent diet) will substantially improve liver enzymes and in a small pilot study of fructose reduction, we documented a trend towards improved ALT[52].

Other studies have suggested associations between NAFLD and fructose by documenting increased soda consumption in those with NAFLD[17], increased uric acid levels (a marker of fructose intake) in children with NAFLD[53] and increased fibrosis with increased fructose consumption in adults with NAFLD[54].

Several other potential limitations of our study should be noted. We studied a high dose of fructose that is not likely to be consumed on a daily basis by most children. We chose the dose of 33% of total calories because in the highest consumers of added sugars among children, 33% of the diet often comes from sugars[55], although it would more likely be a blend and not pure fructose. In addition, a similar study conducted in adults found this dose to be useful in examining the short term response to fructose[27]. Further studies will be needed to test if lower doses of fructose are tolerated in children with NAFLD. Although we controlled the meal on the night before the two visits, the usual diets of our subjects in the days prior to the study could be influencing their response to the study beverages. We asked subjects to continue a typical diet and not change between visits, however variations could exist. Finally, in our healthy control group, several of the children were overweight. We enrolled overweight children as well as normal to improve the chances that we were identifying differences based on the presence of NAFLD and not just elevated BMI. We were unable to enroll all overweight matched controls because most of the overweight children screened for the study had elevated fat % by MRS.

Strengths of this study include the well-controlled methodology including controlling the meal the night prior to the day of the study, utilizing same subject control and comparing to matched controls. Previous studies of dietary sugars in NAFLD have been cross-sectional or case-based association studies, and this is the first study that we know of examining the response to fructose and glucose in both children and in NAFLD. Additionally, previous studies of lipids in pediatric NAFLD have focused on one time fasting levels. To our knowledge, this is the first study of lipid patterns over 24 hours in children with NAFLD and demonstrates important dysregulation of lipid metabolism.

Conclusions

In summary, we demonstrated that dietary fructose induces a marked elevation of TG and decrease in HDL in children with NAFLD; further, although healthy children appear more tolerant of fructose, they also demonstrated the same adverse metabolic effects. Because post prandial TG elevation and HDL levels are important in the pathogenesis of atherosclerosis, these findings suggest that high fructose consumption levels would likely contributing to the increased cardiovascular disease risk found in NAFLD patients. Although we did not find an effect of fructose on NEFA levels, we demonstrated that NEFA is significantly dsyregulated in children with NAFLD and further research is needed to investigate the role of NEFA in pediatric NAFLD. Our data add to the evidence supporting public health recommendations for all children and adolescents to moderate intake of added sugars (the primary source of fructose). Although larger studies are needed, habitual reduction of dietary fructose may be particularly important in

children with NAFLD and other insulin resistant syndromes to potentially reduce adverse effects on lipid cardiovascular disease risk factors.

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Table III-1. Baseline anthropomorphic and metabolic characteristics of study population

Parameter	**non-NAFLD (n=10)**	**NAFLD (n=9)**	**p-value**
Age, year	13.8 (0.7)	13.6 (0.9)	0.834
Male, n(%)	9 (90.0)	8 (88.9)	0.937
Hispanic, n(%)	7 (70.0)	6 (66.7)	0.876
BMI z-score	1.0 (0.3)	2.2 (0.1)	**0.003**
Hepatic fat %	0.6 (0.4)	21.3 (1.8)	**<0.001**
ALT (IU/L)	23.8 (5.0)	138.4 (25.2)	**<0.001**
AST (IU/L)	26.7 (1.7)	84.5 (17.2)	**<0.001**
TC (mg/dL)	157.3 (5.7)	180.1 (10.8)	**0.022**
TG (mg/dL)	99.6 (16.3)	159.1 (25.8)	0.063
HDL (mg/dL)	37.5 (3.3)	36.2 (2.1)	0.757
LDL (mg/dL)	100.4 (4.6)	120.7 (10.0)	0.073
NEFA (mEq/L)	1.2 (0.1)	1.5 (0.2)	0.156
apoB (mg/dL)	73.8 (3.6)	93.3 (7.9)	**0.034**
Glucose (mg/dL)	102.4 (3.6)	99.2 (6.8)	0.678
Insulin (μU/mL)	9.6 (3.3)	39.5 (9.3)	**0.001**
hs-CRP (mg/L)	0.5 (0.2)	3.1 (1.1)	**0.008**
HOMA-IR	2.4 (0.7)	9.6 (2.4)	**0.015**

Values are expressed as mean (SE), and statistical significance is considered as $p<0.05$, calculated by independent two sample t-test. ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; NEFA, non-esterified fatty acid; apoB, apolipoprotein B; hs-CRP, high-sensitivity C-reactive protein; HOMA-IR, homeostatic model assessment for insulin resistance, calculated by (glucose*insulin)/405.

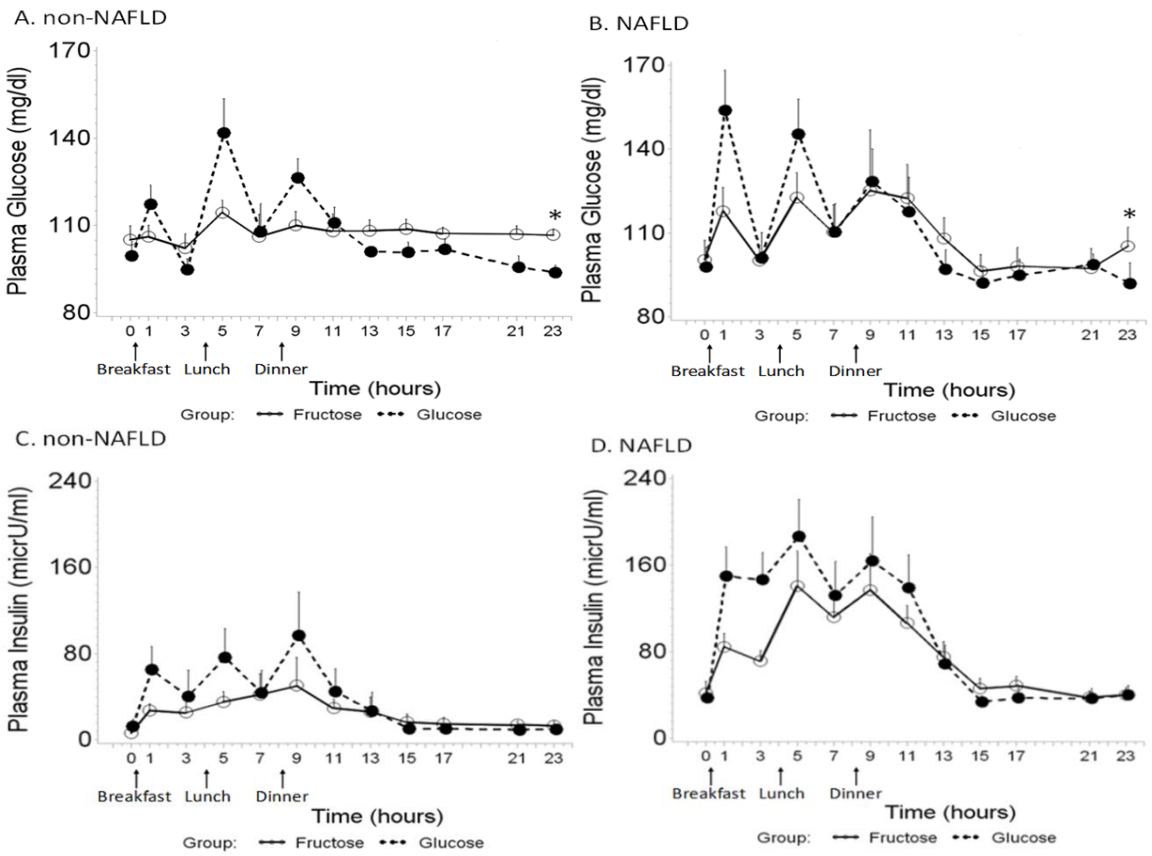

Figure III-1. Twenty-four hour mean plasma glucose (A and B) and insulin (C and D) concentration by consumption of three consecutive meals with fructose beverages (solid lines) and glucose beverage (dashed lines) in non-NAFLD controls (A and C) and pediatric NAFLD (B and D). Error bars stand for SE. In non-NAFLD subjects, the 24h-IAUC of insulin was significantly increased after consuming glucose beverage as compared to fructose beverage (mean ± SE: 591.4 ± 239.9 vs. 451.6 ± 228.6, p=0.021), but there was no significant differences in NAFLD. The 24h-IAUC of plasma insulin was significantly higher in NAFLD subjects as compared to non-NAFLD controls during both fructose consumption (mean ± SE: 996.3 ± 189.8 vs. 451.6 ± 228.6, p=0.028) and glucose consumption (mean ± SE: 1444.9±247.8 vs. 591.4±239.9, p=0.008). Although the difference of 24h-IAUC of plasma glucose did not reach the statistical significance, at the end of fructose beverage feeding (time point of 23h), fasting plasma glucose remained significantly higher as compared to that after one day glucose beverage feeding, for both NAFLD (p=0.046) and non-NAFLD (p=0.008) [marked with *].

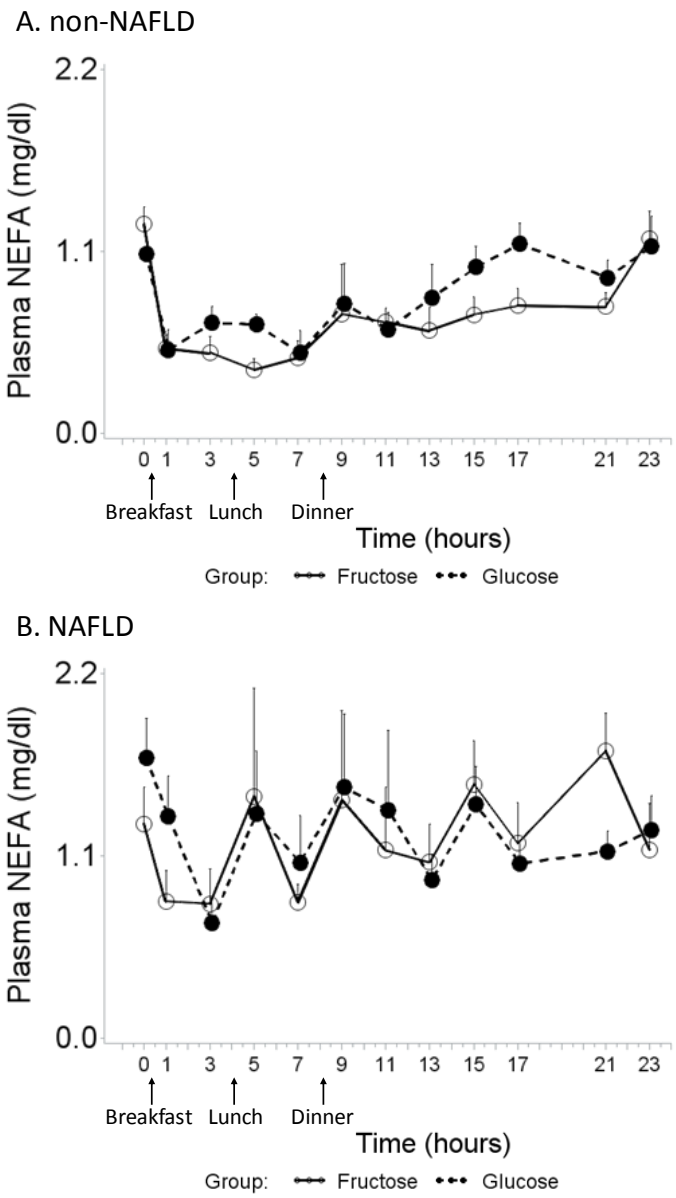

Figure III-2. Twenty four hour plasma NEFA profile after consumption of fructose beverages (solid line) and glucose beverages (dashed line) in non-NAFLD controls (A) and pediatric NAFLD (B). Error bars stand for SE. In NAFLD children, an overall increased levels of plasma NEFA was observed compared to non-NAFLD controls, and there was no difference between fructose beverage and glucose beverage feeding period (mixed model, beverage: $p = 0.260$; disease: $p = 0.002$; beverage x disease interaction: $p = 0.558$).

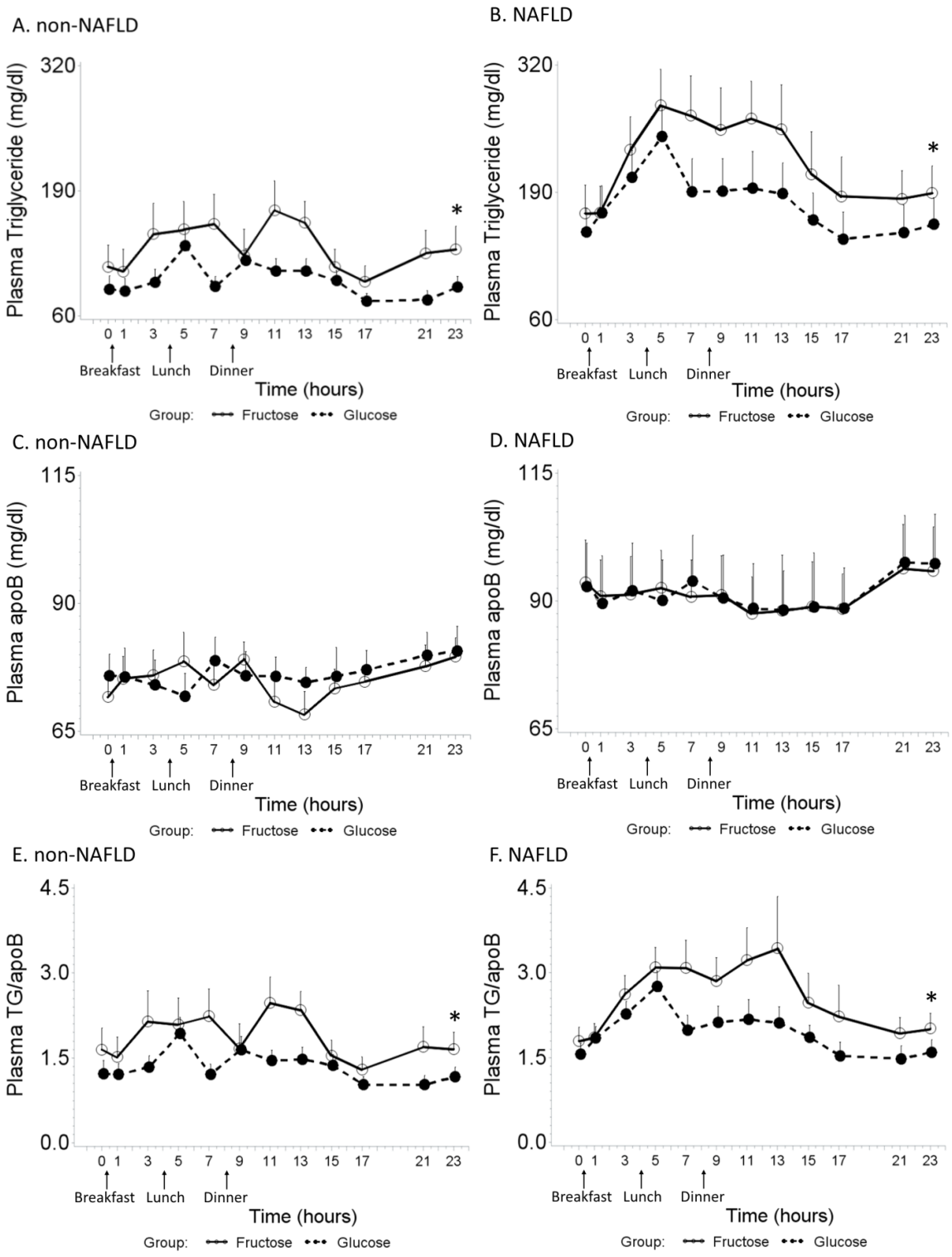
A. non-NAFLD
B. NAFLD
C. non-NAFLD
D. NAFLD
E. non-NAFLD
F. NAFLD
Plasma Triglyceride (mg/dl)
Plasma apoB (mg/dl)
Plasma TG/apoB
320
190
60
115
90
65
4.5
3.0
1.5
0.0
0 1 3 5 7 9 11 13 15 17 21 23
Breakfast
Lunch
Dinner
Time (hours)
Group: Fructose Glucose
*

Figure III-3. Twenty four hour TG (A and B), apoB (C and D), TG/apoB (E and F) profile during consuming fructose beverage and glucose beverage in non-NAFLD controls (A, C and E) and pediatric NAFLD (B, D, and F). Error bars stand for SE. **A-B)** The 24-hr IAUC of plasma TG was significantly increased by consumption of fructose beverage in contrast to glucose beverage, for both children with NAFLD (mean±SE: 1421.2±267.1 vs. 757.3±136.5, p=0.011) and children without NAFLD (mean±SE: 622.4±110.2 vs. 325.7±87.6, p=0.027); and NAFLD exacerbated this fructose effect on plasma TG (p=0.019). At the end of visit (time point of 23h), fasting plasma TG in the next morning was significantly elevated after fructose beverage compared with glucose beverage for both NAFLD (p=0.011) and non-NAFLD (p=0.005) subjects [marked with *]. **C-D)** There was no significant difference in plasma apoB under two beverage feeding conditions within 24 hours, in both non-NAFLD and NAFLD subjects. **E-F)** Compared to glucose beverage, fructose beverage consumption was associated with greater postprandial response in TG/apoB, for both NAFLD (p<0.0001) and non-NAFLD (p=0.030), and NAFLD exacerbated the effect of fructose (p=0.018) on TG/apoB (mixed model, beverage: p<0.0001; disease: p=0.045; beverage x disease interaction: p=0.025). At the end of visit (time point of 23h), fasting plasma TG/apoB in the next morning was significantly elevated after fructose beverage compared with glucose beverage for both NAFLD (p=0.011) and non-NAFLD (p=0.016) subjects [marked with *].

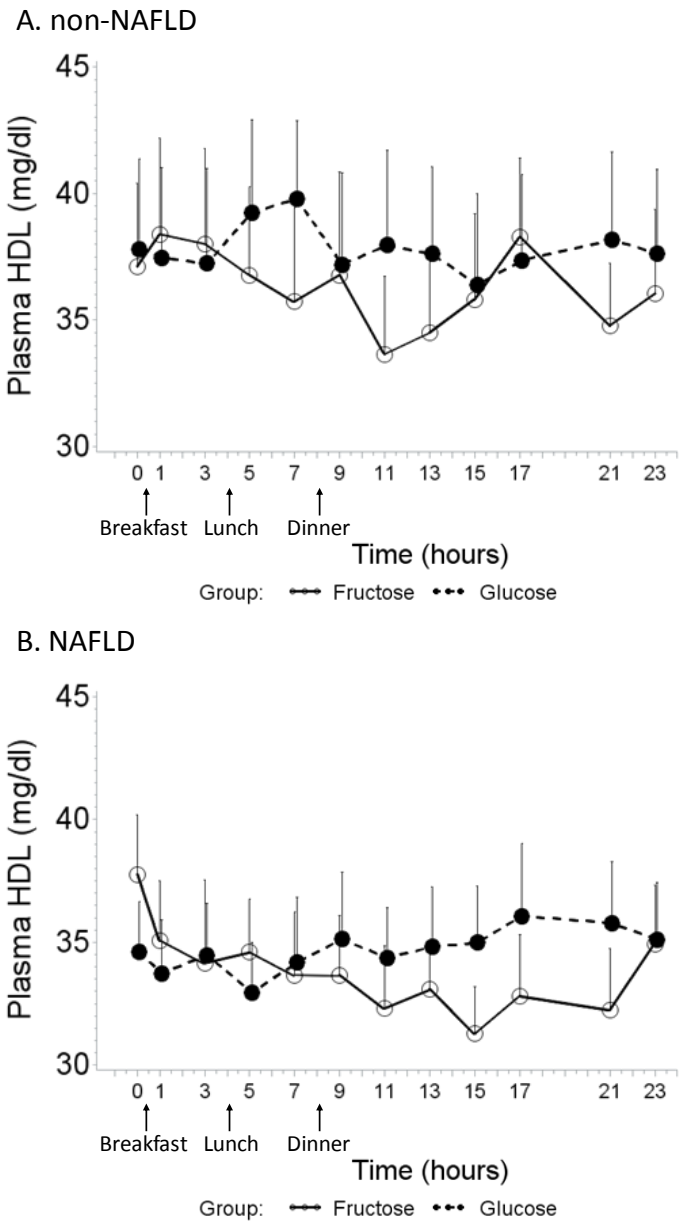

Figure III-4. HDL cholesterol trend over twenty four hours after consumption of fructose beverage (solid lines) and glucose beverages (dashed lines) in non-NAFLD controls (A) and pediatric NAFLD (B). Error bars stand for SE. HDL had an overall decline during consuming fructose beverage as compared to glucose beverage, and no difference in HDL response was found between NAFLD and non-NAFLD subjects (mixed model, beverage: p=0.0006; disease: p=0.478; beverage x disease interaction: p=0.382).

Chapter IV. Dietary Fructose Reduction Improves Markers of Cardiovascular Disease Risk in Adolescents

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Short title: Fructose and cardiovascular risk (Jin et al)

Subject code: [101] Nutrition; [135] Risk Factor

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) has emerged as the most common liver disease in adolescents. Cardiovascular complications are a leading cause of mortality in NAFLD. Fructose, a common nutrient in the westernized diet, has been reported to be associated with increased cardiovascular risk, but its impact on adolescents with NAFLD is not well understood.

Methods and Results: This was a 4-week randomized, controlled, double-blinded intervention study using calorie-matched fructose- or glucose-only beverages designed for this study. Twenty-four overweight and obese Hispanic-American adolescents who had hepatic steatosis >5% on imaging and who were regular consumers of sweet beverages were enrolled and evaluated at 0, 2 and 4 weeks. After replacing their usual sweet beverages with study-provided glucose-only beverages for 4 weeks, there was no change in hepatic steatosis; however adipose insulin sensitivity, high sensitivity C-reactive protein (hs-CRP), and low-density lipoprotein (LDL) oxidation all improved significantly.

Conclusions: These findings demonstrate that reduction of fructose improves several important factors in the mechanism of cardiovascular disease despite a lack of measurable improvement in hepatic steatosis. Reducing dietary fructose may be an effective intervention to blunt atherosclerosis progression among NAFLD patients and should be evaluated in longer term clinical trials.

Clinical Trial Registration: clinicaltrials.gov, NCT01188083

Key words: nonalcoholic fatty liver disease, cardiovascular disease, fructose, lifestyle, obesity,adolescents

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common, obesity-associated chronic liver disease. Estimates of the NAFLD prevalence range from 3 to 36% of the general American population, with increased risk in patients with obesity and patients with type 2 diabetes mellitus[1]. The increasing number of children with NAFLD is of considerable public health concern. Pediatric NAFLD has more than doubled in the past two decades and currently affects an estimated 11% of adolescents in the United States[2]. The ethnic disparity is presented in the prevalence of pediatric NAFLD with Hispanics being mostly affected[2]. Studies in adults have demonstrated that NAFLD is closely associated with cardiovascular disease (CVD)[3-5]. NAFLD is suspected to exacerbate the pathogenesis of CVD through the systemic release of inflammatory and oxidative-stress mediators and through insulin resistance and atherogenic dyslipidemia[6]. Although longitudinal studies in pediatric patients with NAFLD are not available to examine long-term CVD risk, emerging evidence suggests that adolescents with NAFLD already have increased subclinical atherosclerosis[7-9]. In view of the high prevalence of NAFLD among adolescents and the strong association between NAFLD and CVD, exploring early prevention strategies through diet and lifestyle modification and developing effective treatment options are clearly needed.

One of the culprits in the development of NAFLD in adolescents has been suggested to be high consumption of fructose. Fructose is a widely used sweetener in beverages and many processed foods and its consumption has increased dramatically over the last 40

years[10]. Recent data have shown that fructose intake represents more than 12% of daily calories consumed by U.S. adolescents (primarily from sweet beverages)[11], which exceeds the current recommendations[12,13]. In both animal and short-term human feeding studies, fructose increases hepatic fat accumulation through insulin resistance, *de novo* lipogenesis, and plasma hypertriglyceridemia[14-16]. Fructose also increases oxidative damage by reducing antioxidant defenses and enhancing the production of reactive oxygen species (ROS)[17-19]. This combination of lipid overload and oxidative stress makes fructose suspect in the development and progression of NAFLD[20,21]. Furthermore, fructose-induced dyslipidemia, insulin resistance, and oxidative damage could specifically contribute to the increased CVD risk seen in NAFLD. However, direct evidence showing the benefits of fructose restriction on hepatic steatosis or CVD risk in NAFLD is still lacking, especially in adolescents, a group characterized by both high prevalence of NAFLD and high intake of fructose[2,11].

In the current study, we recruited overweight and obese Hispanic-American adolescents with frequent consumption of sweet beverages and elevated hepatic fat (> 5%) as measured by state-of-the-art magnetic resonance spectroscopy (MRS) methodology [22]. In a randomized, calorie-matched, controlled study, we examined whether hepatic steatosis and associated cardiovascular risk factors would be improved after 4 weeks of fructose reduction achieved through replacement with glucose-only beverages.

Methods

Subjects and study design: This was a 4-week, double-blinded, randomized-controlled intervention study. Overweight and obese (BMI z-score ≥ 85th percentile) adolescents were recruited from pediatric clinics at Emory Children's Center and from nearby community centers through flyers and presentations at community events. Eligibility criteria included self-identification as Hispanic, ages 11-18 years; BMI ≥ 85th percentile for age and gender; and average self-reported consumption of at least 3 servings of sweet beverages per day. Sweet beverages were defined as drinks sweetened with added sugars (eg, sodas, sweet tea, sport drinks, flavored drinks) or naturally sweet beverages (eg, 100% fruit juice), but did not include artificially-sweetened drinks. Although 100% juice is not typically included in assessment for sugar consumption, we included it because the fructose content of juice is high and there is little evidence to suggest that it would not have the same effect as fructose from sodas or other sweetened beverages. Exclusion criteria included known liver diseases; diabetes or fasting glucose > 126 mg/dl; renal insufficiency (creatinine > 2 mg/dl); any chronic disease requiring daily medication; acute illness within past 2 weeks prior to enrollment (defined by fever > 100.4ºF); and supplement and/or anti-oxidant therapy within 4 weeks prior to enrollment.

All recruited participants underwent MRS to quantify hepatic fat; subjects with hepatic fat > 5% were considered as having hepatic steatosis[23]. Subjects with hepatic steatosis were randomized within 1 week of the baseline MRS to either study-provided fructose- (considered as fructose continuation) or glucose-containing (considered as fructose

reduction) beverage groups; and follow-up visits were completed at 2 and 4 weeks after randomization. The interim visit at 2 weeks after randomization was intended to assess safety and to promote compliance and was not a primary outcome. Prior to each study visit, subjects were required to fast for at least 12 hours and fasting blood samples were collected for the laboratory measurements between 7 and 9 am. Initially, a total of 24 adolescents with confirmed hepatic steatosis by MRS agreed to participate in this randomized 4-week beverage intervention trial. At the 2-week visit, one subject's serum alanine aminotransferase (ALT) increased to >10 times the upper limit of normal and participation was terminated for safety concerns. Two subjects declined to return after the 2 week visit and dropped out. The remaining 21 subjects successfully completed the study protocol.

During the 4-week study, participants were instructed to drink 3 servings (12 fl oz bottles) of study-provided beverages each day. The study beverages contained 33 grams of sugar (standard amount of sugar in a typical soda) in the form of either glucose or fructose and were matched for color and flavoring (Power Brands, Beverly Hills, CA). Each subject was given a sufficient supply of study beverages to take home or beverages were delivered directly to their house by the research coordinator. Participants and investigators were blinded as to the contents of the drinks. No other sugar-containing beverages were allowed during the study period. Subjects were requested not to change their diet pattern and physical activity. Compliance was monitored through daily drink logs, return of empty beverage bottles at each study visit, and weekly phone calls from the study coordinator. The study protocol was approved by the Emory University and

Children's Healthcare of Atlanta IRBs and written informed consent (parental consent obtained for subjects <18 years) and assent (when applicable) were obtained for each subject prior to participation in the study.

Determination of hepatic fat: Hepatic fat was assessed by MRS using our previously described methods[22]. Briefly, we used a rapid 15-sec acquisition technique obtained during a single breath hold. The sequence is constructed from five concatenated echoes using a fixed set of echo times (TE) (12, 24, 36, 48, and 72ms), with each echo having a repetition time (TR) = 3000ms, voxel = 3 x 3 x 3 cm^3, 1024 points, and 1200 Hz bandwidth. The acquisition was repeated three times for reproducibility. Data were exported off-line for automatic processing with in-house software (Matlab, Mathworks, Natick, MA). Water and lipid magnitude spectra were analyzed by determining the area under the curve (AUC) corresponding to a user-defined frequency range surrounding the corresponding water/lipid peaks (water peak: 4.6ppm; lipid peak: 1.3, 2.0ppm). The integrated magnitude signals at each TE were fit to exponential T2 decay curves, whereby the equilibrium signal (M_0) and the relaxation rate (R2 = 1/T2) were determined by least-squares regression approximation. Using M_0 for water and lipid, the T2-corrected hepatic lipid fraction was calculated from: % Hepatic Lipid = M_{0lipid} / (M_{0lipid} + M_{0water}).

Laboratory analyses: Fasting blood samples were collected into EDTA-coated tubes and plasma was separated immediately. Plasma samples were protected from light and

transported on ice to the laboratory for further processing (within 4 hours). All lipid measurements were performed by the Emory Lipid Research Laboratory using AU480 chemistry analyzer (Beckman Coulter). Total cholesterol and triglycerides were measured by enzymatic method using reagents from Beckman (Beckman Diagnostics, Fullerton, CA). Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured by homogeneous enzymatic assay (Sekisui Diagnostics, Exton, PA). Free fatty acid (FFA) and glucose was quantified by colorimetric method (Sekisui Diagnostics, Exton, PA). Insulin and high sensitivity C-reactive protein (hs-CRP) were assessed using immunoturbidometric method (Sekisui Diagnostics, Exton, PA). Plasma level of oxidized LDL (oxLDL) was quantified using Mercodia human oxLDL ELISA kit (Winston-Salem, NC) which is based on the 4E6 antibody against oxidized apolipoprotein B (apoB). Baseline and week 4 serum plasminogen activator inhibitor-1 (PAI-1) concentrations were assessed using an ELISA kit (abcam, Cambridge, MA). Very low density lipoprotein (VLDL) particle numbers and sizes were measured by nuclear magnetic resonance (NMR) spectroscopy using a 400-MHz proton analyzer (LipoScience Inc., Raleigh, NC) as described elsewhere[24]. Serum ALT and aspartate aminotransferase (AST) were measured by the Emory University Hospital Medical Laboratory.

Insulin resistance index: Insulin resistance was assessed by the homeostasis model of assessment - insulin resistance (HOMA-IR) and the newly defined adipose IR index[25]. HOMA-IR was calculated by glucose (mmol/L) x insulin (pmol/L) / 22.5 at fasting state, and adipose IR index was calculated as fasting FFA (mmol/L) x insulin (pmol/L).

Measurement of oxidative stress: The *ex vivo* LDL oxidative susceptibility assay was performed using the method previously described by Esterbauer et al[26]. Freshly collected plasma samples (2mL) were adjusted with high density solution of NaBr containing 0.1% EDTA to density of 1.21 g/ml and subjected to ultracentrifugation at 39,000 RPM for 48 hours (15°C) using the 50.4Ti rotor (Beckman Instruments, Palo Alto, CA). The supernate (d < 1.21 g/ml) was fractionated into VLDL, LDL, and HDL by fast protein liquid chromatography (FPLC, Pharmacia) as previously described[27]. The oxidative susceptibility of LDL was assessed by continuous monitoring of the formation of conjugated dienes during the lipid peroxidation at the absorbance of 234 nm with the DU530 spectrophotometer (Beckman). Briefly, 40µg LDL cholesterol in 0.01 M PBS without EDTA was exposed to 9µM Cu_2SO_4 and the kinetics of lipid peroxidation was monitored for a total of 300 min at the interval of 1 min at room temperature. Lag time, an indicator of oxidative susceptibility, was defined graphically as the time (min) to the initiation of oxidation. LDL with longer lag phase would be more resistant to oxidative modification and is described as having low oxidative susceptibility. To minimize inter-assay variability, LDL preparations from each participant collected at baseline and week 4 were assayed in the same batch.

Statistical analyses: Statistical analyses were performed using SPSS (version 17.0, Chicago, IL). Results in the tables were reported as mean (SD) unless indicated otherwise. Statistical significance was considered as $p \leq 0.05$. Data were examined for normality and equal variance prior to any analyses. Baseline differences between two study beverage groups were examined using the Mann-Whitney test and the gender difference

was evaluated with Fisher's Exact test. Changes from baseline to week 4 within each beverage group were compared by Wilcoxon test, and the differences in % change over the 4 weeks between two beverage groups were assessed by conducting Mann-Whitney test. Our power analysis anticipated that by using 3% as a mean change of hepatic fat and standard deviation from pilot data, greater than 90% study power would be achieved by recruiting 6 subjects in each beverage group. We aimed to recruit 12 subjects into each group to allow for potential drop-out and variable responses to sugar between individuals.

Results

The study design is summarized in **Figure 1**. Baseline characteristics of participants assigned to the two intervention groups are presented in **Table 1**. There were no significant differences in age, gender, weight, glycemic status, and lipid profile between the two groups. The baseline mean values of serum ALT and AST appear greater in the glucose beverage group because the data were skewed by one subject who had high values (ALT: 373 U/L; AST: 214 U/L). When this subject was excluded from analysis, the mean values of ALT and AST were 32.7 ± 17.4 (mean ± SD, range from 16 – 69 U/L) and 33.8 ± 6.98 (mean ± SD, range from 24 – 43 U/L), respectively.

As shown in **Table 2**, after the 4-week intervention, subjects receiving glucose-containing beverages (considered as fructose reduction) had significant improvement in plasma hs-CRP ($p = 0.028$), adipose IR index ($p = 0.004$), and plasma FFA ($p = 0.027$) as compared to baseline. In addition, they had a significant reduction in circulating oxLDL

levels (p = 0.034). The susceptibility of LDL to Cu^{2+}-induced oxidative modification was significantly improved as demonstrated by a 2-fold increase in LDL lag time (p = 0.010). In the group receiving fructose-containing beverages (considered as fructose continuation), there was a rising trend in adipose IR index from baseline to week 4, although the p-value did not reach statistical significance (p=0.21). In contrast to the glucose group, there were no significant changes in plasma FFA, hs-CRP, oxLDL levels or the ex vivo oxidative susceptibility of LDL. For both groups, there were no significant changes in body weight, hepatic fat, liver enzymes, fasting TG, and PAI-1 levels after 4 weeks.

We also compared the percent changes in all indicators over 4 weeks between the glucose- and fructose-containing beverage groups. Adolescents who were randomized to glucose-containing beverages had significant improvements in hs-CRP (-8.07% vs. 23.8%, p = 0.019) and adipose IR index (-27.2% vs. 47.0%, p =0.028) as compared to those who continuously consumed fructose-containing beverages (**Figure 2. A,B**). Furthermore, based on NMR spectroscopy, the percent change of large VLDL particles from baseline to week 4 was significant between the two beverage groups (p = 0.041), with the particle number increased by 53.9% for the individuals randomized to fructose-containing beverages and decreased by 5.73% in glucose-containing beverage group (**Figure 2. C**).

Discussion

NAFLD affects almost one-third of overweight adolescents and has long-term health consequences, particularly from CVD. Some have described NAFLD as the hepatic manifestation of metabolic syndrome because of its frequent association with insulin resistance, increased visceral adiposity, and atherogenic dyslipidemia[28]. Even in childhood, patients with NAFLD demonstrate atherosclerotic lesions as measured by increased carotid intima media thickness (cIMT)[9]. Thus, adolescents with NAFLD are believed to be at high risk of future CVD, and studies are needed that examine modifiable influences contributing to increased cardiovascular risk in NAFLD. In this study, we examined Hispanic-American adolescents because of their dramatically increased risk of NAFLD[29,30]. We performed a calorie-matched, randomized, controlled reduction of fructose in order to isolate the effect of the sugar type from calories added by sweet beverages. We found that fructose reduction through replacing with glucose-only beverages resulted in an improved cardiometabolic profile, including increased insulin sensitivity, reduced hs-CRP, fewer large VLDL particles and fewer oxidized LDL. This combination of improvements in a carefully-controlled randomized trial indicates that fructose plays a unique role in the increased CVD risk seen in NAFLD patients.

Several possible mechanisms may explain the metabolic improvements that were observed in this study. Fructose consumption has been shown to increase visceral adipose tissue (VAT) in both animal and human studies[20]. Increased VAT acts as a metabolically active tissue that produces numerous inflammatory cytokines such as hs-CRP, tumor

necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) and promotes both hepatic and systemic insulin resistance[1]. On the other hand, fructose has been shown to affect the microbiome and increase endotoxin release into the circulation[14,31]. Increased endotoxin release activates the innate immune system and inflammation, subsequently exacerbating insulin resistance[32]. Impaired insulin sensitivity, in combination with increased VAT, would further dysregulate lipid metabolism, resulting in excess FFA flux from the peripheral tissue returning to the liver and thereby increased hepatic secretion of large, more atherogenic VLDL[33,34]. Consistent with these pathways, in our current study, by reducing fructose consumption for 4 weeks, adolescents with hepatic steatosis showed improved inflammation and adipose insulin sensitivity as indicated by hs-CRP and adipose IR index, along with improved FFA levels and large VLDL particle concentrations in the circulation.

Improvement in oxidative stress may also result from reduced fructose ingestion. We have previously shown that oxLDL improves in response to a low fructose diet over 6 months[35]; however, in that experiment, the subjects made multiple changes in their diets. In this trial, we blinded participants and investigators to the assigned study-provided beverages to minimize the chance that other dietary changes were responsible for the effects. We again found a significant decrease in circulating oxLDL levels and expanded on this by testing LDL susceptibility to oxidation by reducing fructose consumption. Previous work has demonstrated that fructose-rich diets (10% in drinking water) decreased oxidation resistance of lipoprotein fractions in copper-induced lipoperoxidation in a rat model[36], which was consistent with our new finding in adolescents with hepatic

steatosis. This impaired oxidation resistance could possibly reflect the diminished endogenous antioxidant content of the lipoprotein particles and the increased production of free radicals, as shown in animal studies[17-19]. Although consistent in animal studies[37], fructose-induced oxidative stress has not been well demonstrated in human trials and need to be carefully examined in the future.

We also expected that fructose reduction for 4 weeks would improve hepatic steatosis and PAI-1. Hepatic fat has been shown by others to increase after soda consumption over 6 months[38]. A recent pilot study of fructose reduction in adults with NAFLD indicated a decline in intrahepatic fat content along with weight loss after 6 months[39]. In our study, neither group had a change in hepatic steatosis levels. While this disproved our hypothesis, our study was designed to be eucaloric. The fructose reduction associated with loss of hepatic fat in adults was in the setting of weight loss and this might be a requirement for removing excess stored energy in the liver. Further, since hepatic steatosis may be a downstream result of worsened insulin resistance, visceral fat accumulation, and oxidative stress, it could be that a longer study would have resulted in changes in the liver. We also tested PAI-1 because it is a coagulant factor that has been found to be strongly associated with hepatic steatosis[40,41]. Increased PAI-1 has been proposed as an underlying mechanism potentially responsible for accelerated atherogenesis in NAFLD[6]. Given the proposed relationship between PAI-1 and steatosis, it is consistent that PAI-1 also did not change in either group. It also suggests that PAI-1 is less related to insulin resistance, as it did not change in parallel with the changes in insulin resistance.

There are some limitations of our pilot study. As an outpatient study, we did not control the entire diet in our study subjects over the intervention period. This is not practical for children and would likely be less applicable to health recommendations. However, body weights of the subjects remained stable from baseline to the end of the study, which implies a eucaloric period. We asked subjects to replace their usual consumption of sweet beverages with the study-provided beverages to minimize other changes in the diet. The 4-week study period was brief compared to the years that adolescents typically consume sweet beverages and a longer study would be helpful to confirm the persistence of the improvements and determine if a longer reduction would also improve liver findings. We selected 4 weeks based on other studies and in part to improve compliance. We studied a group of Hispanic adolescents because of their disparate risk for NAFLD and future CVD. These findings are critical for this particular group; however they may not be generalizable to NAFLD patients from other ethnic backgrounds. Finally, due to the sample size, sex and puberty differences in response to the fructose reduction could not be examined in our cohort.

In summary, this double-blind, randomized controlled study comparing glucose beverages to fructose beverages demonstrates that reduction of fructose for 4 weeks in adolescents with hepatic steatosis (consistent with NAFLD) improves adipose insulin sensitivity, inflammation, plasma FFA, and LDL oxidation. These data suggest that treatment strategies targeting fructose reduction would reduce future cardiovascular risk in adolescents with NAFLD. Longer studies of fructose reduction and fructose reduction studies utilizing non-invasive vascular measurements of atherosclerosis will be needed to

prove this. In the meantime, given that fructose reduction is an inexpensive and side-effect free intervention for patients with NAFLD, it can be considered as an adjunct to current therapies.

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Disclosures

The authors of the study have no conflicts of interest to declare.

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Table IV-1. Baseline characteristics of participants enrolled in the 4-week beverage intervention trial

Parameters, mean (SD)	**Fructose (n=9)**	**Glucose (n=12)**
Age (years)	14.2 (2.64)	13.0 (2.45)
Male, n (%)	3 (33.3)	8 (66.7)
Body weight (kg)	82.3 (16.9)	82.0 (14.8)
BMI z-score	2.25 (0.57)	2.15 (0.31)
Hepatic fat (%)	14.5 (5.36)	14.0 (6.12)
ALT (U/L)	33.0 (20.2)	61.1 (99.6)
AST (U/L)	32.4 (9.18)	48.8 (52.4)
Triglycerides (mmol/L)	1.77 (1.19)	1.78 (0.69)
Cholesterol (mmol/L)	4.34 (0.71)	4.40 (1.18)
LDL (mmol/L)	2.79 (0.81)	2.87 (1.00)
HDL (mmol/L)	1.19 (0.25)	1.12 (0.22)
FFA (mmol/L)	0.97 (0.23)	1.11 (0.47)
Glucose (mmol/L)	5.49 (0.82)	5.01 (1.28)
Insulin (pmol/L)	211 (89.3)	244 (206)
HOMA-IR (mmol/L·pmol/L)	50.9 (20.0)	57.8 (56.8)
adipose IR (mmol/L·pmol/L)	199 (78.7)	240 (189)
hs-CRP (mg/L)	6.78 (9.47)	5.21 (4.63)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FFA, free fatty acid; HOMA-IR, homeostatic model assessment for insulin resistance index, calculated as fasting glucose (mmol/L) x insulin (pmol/L)/22.5; Adipose IR, adipose insulin resistance index, calculated as fasting FFA (mmol/L) x insulin (pmol/L); hs-CRP, high sensitivity C-reactive protein

Table IV-2. Liver measurement, lipid profile, glycemic status, and inflammation and oxidation status at baseline and week 4 after consumption of study provided fructose- or glucose-containing beverages in adolescents with hepatic steatosis

Parameters, mean (SD)	Fructose (n=9)			Glucose (n=12)		
	Baseline	Week 4	p-value	Baseline	Week 4	p-value
Body weight (kg)	82.3 (16.9)	83.0 (17.6)	0.123	82.0 (14.8)	82.5 (14.4)	0.346
Hepatic fat (%)	14.5 (5.36)	13.6 (5.50)	0.326	14.0 (6.12)	13.8 (6.64)	0.732
ALT (U/L)	33.0 (20.2)	33.4 (13.2)	0.678	61.1 (99.6)	59.7 (91.3)	0.969
AST (U/L)	32.4 (9.18)	33.3 (10.0)	0.953	48.8 (52.4)	47.7 (51.9)	0.430
Triglycerides (mmol/L)	1.77 (1.19)	1.15 (0.36)	0.139	1.78 (0.69)	1.72 (0.80)	0.754
Cholesterol (mmol/L)	4.34 (0.71)	4.01 (0.68)	0.164	4.40 (1.18)	4.32 (0.88)	0.675
LDL (mmol/L)	2.79 (0.81)	2.46 (0.92)	0.325	2.87 (1.00)	2.92 (0.78)	0.762
HDL (mmol/L)	1.19 (0.25)	1.06 (0.23)	0.085	1.12 (0.22)	1.07 (0.29)	0.478
FFA (mmol/L)	0.97 (0.23)	0.90 (0.31)	0.716	**1.11 (0.47)**	**0.78 (0.24)**	**0.027**
Glucose (mmol/L)	5.49 (0.82)	5.16 (0.71)	0.327	5.01 (1.28)	5.20 (0.89)	0.723
Insulin (pmol/L)	211 (89.3)	313 (204)	0.260	244 (206)	198 (117)	0.695
adipose IR (mmol/L·pmol/L)	199 (78.7)	251 (116)	0.214	**240 (189)**	**147 (91.9)**	**0.004**
HOMA-IR (mmol/L·pmol/L)	50.9 (20.0)	74.0 (55.5)	0.441	57.8 (56.8)	44.3 (23.6)	0.754
hs-CRP (mg/L)	6.78 (9.47)	7.06 (7.07)	0.477	**5.21 (4.63)**	**3.99 (3.78)**	**0.028**
LDL lag time (min)	18.6 (12.9)	23.9 (17.3)	0.084	**18.5 (11.0)**	**37.1 (25.2)**	**0.010**
Oxidized LDL (mU/L)	8.74 (2.95)	7.99 (3.33)	0.501	**8.49 (3.22)**	**7.06 (3.18)**	**0.034**
PAI-1 (ng/ml)	47.3 (7.76)	49.5 (7.01)	0.324	51.0 (6.31)	51.2 (7.92)	0.876

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FFA, free fatty acid; Adipose IR, adipose insulin resistance index, calculated as fasting FFA (mmol/L) x insulin (pmol/L); HOMA-IR, homeostatic model assessment for insulin resistance index, calculated as fasting glucose (mmol/L) x insulin (pmol/L)/22.5; hs-CRP, high sensitivity C-reactive protein; PAI-1, Plasminogen activator inhibitor-1

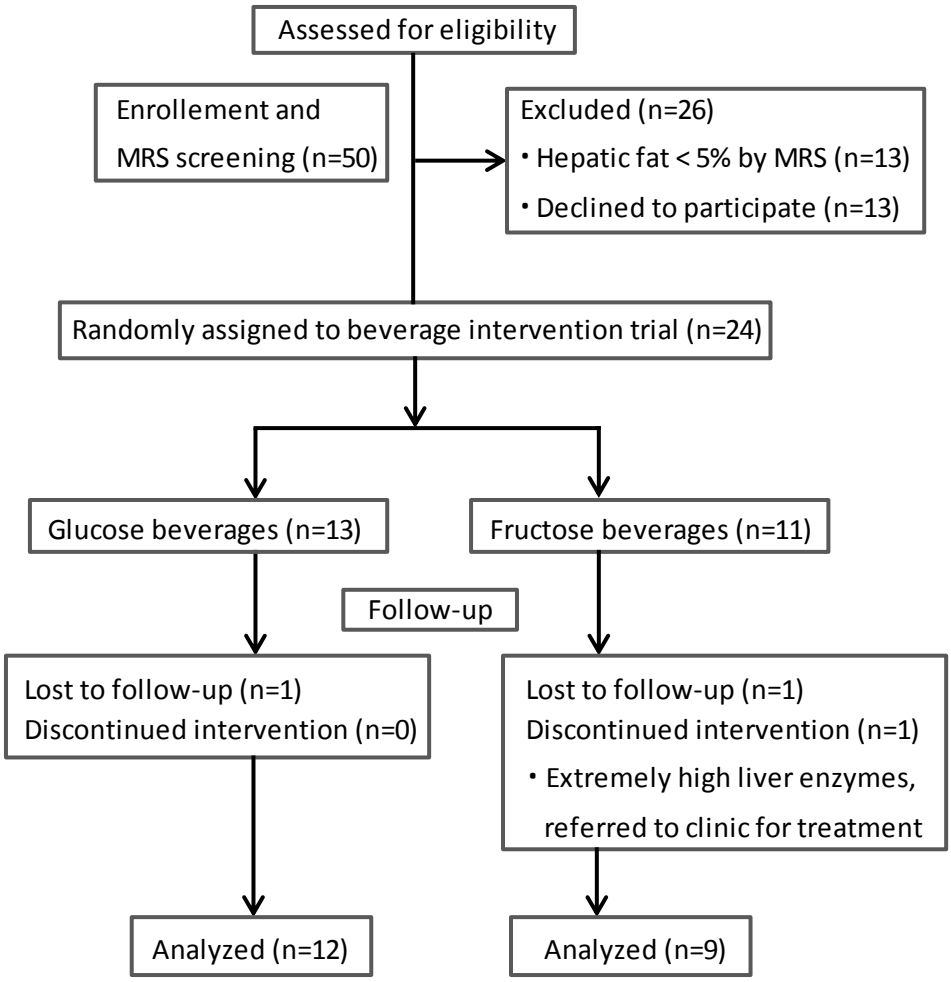

Figure IV-1. Study design for the randomized beverage intervention trial.

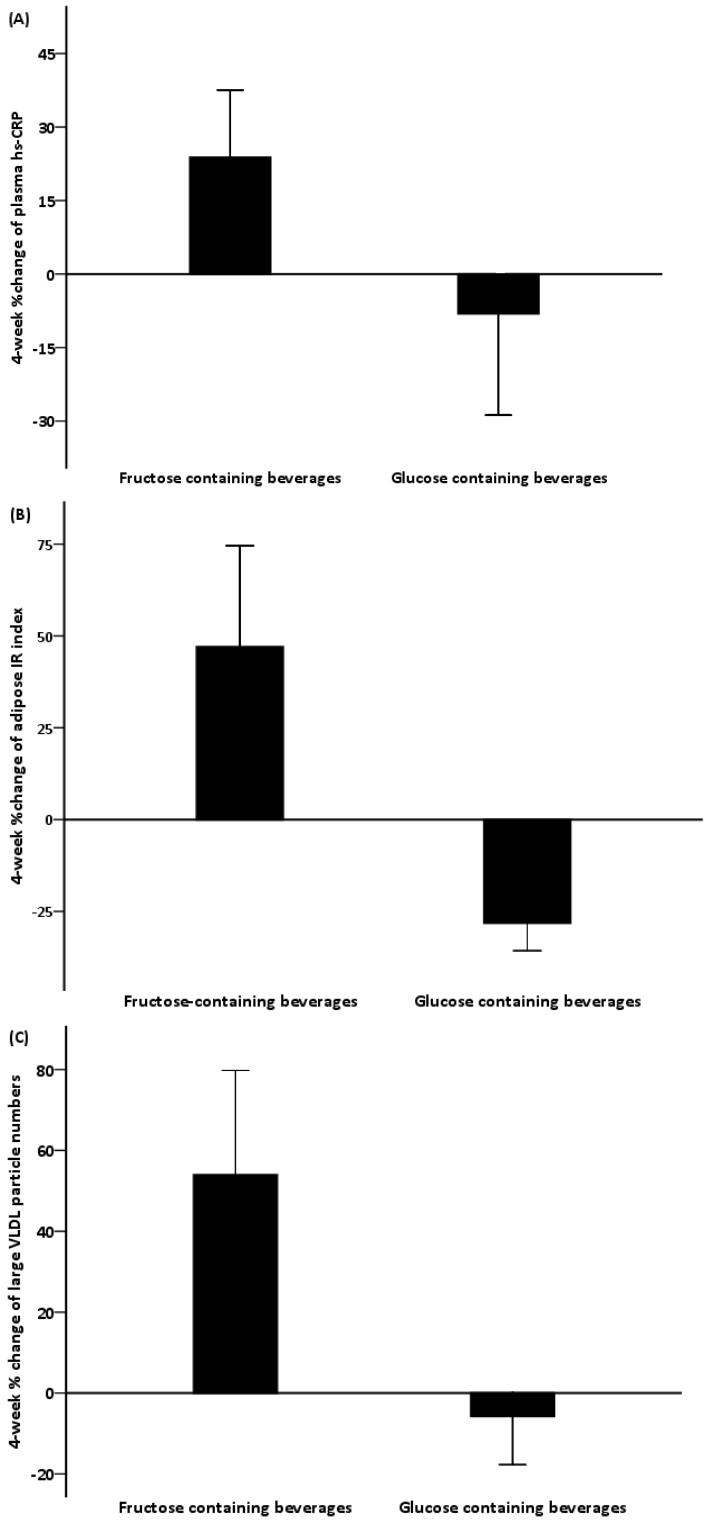

Figure IV-2. 4-week percent changes of plasma hs-CRP (A), adipose IR index (B), and large VLDL particle numbers (C) in both fructose- and glucose-containing beverage groups among adolescents with hepatic steatosis. Error bars stand for SE.

Chapter V: Fructose Induced Endotoxemia in Pediatric Nonalcoholic Fatty Liver Disease

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Keywords: nonalcoholic fatty liver disease, fructose, endotoxin, children and adolescents

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Abstract

Objective: Nonalcoholic fatty liver disease (NAFLD) is frequently obesity-associated, and the most common chronic liver disease in children. The westernized diet has been implicated in the development of NAFLD, particularly the high consumption of fructose. Previous studies have suggested that increased endotoxin is related to obesity, however no studies have previously examined if dietary fructose induces endotoxemia in children with NAFLD.

Design and Methods: We compared fasting endotoxin levels in a cohort of Hispanic-American, obese adolescents who were high fructose consumers, and correlated endotoxin to cytokines and insulin resistance index. Next, we assessed acute alteration of postprandial endotoxin levels in response to fructose beverages in adolescents with and without NAFLD in a well-controlled feeding study, and we evaluated a 4-week fructose reduction on endotoxemia in NAFLD as well.

Results: Hispanic-American adolescents with hepatic steatosis had elevated fasting plasma endotoxin levels as compared to their obese controls, and the increased endotoxin correlated with insulin resistance, tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). Adolescents with NAFLD had an overall increase of postprandial endotoxin levels within 24 hours after consumption of fructose beverages comparing with healthy adolescents. Continuation of fructose beverages for 4 weeks increased endotoxin levels in NAFLD, while calorie-matched glucose-only beverages did not induce significant elevations in endotoxin.

Conclusion: Together, our findings suggest an important role for fructose-induced endotoxin in the development and propagation of NAFLD in pediatric population.

Introduction

In parallel with the epidemic of obesity, nonalcoholic fatty liver disease (NAFLD) has merged as the leading cause of chronic liver disease and currently affects approximately 40% of obese adolescents in the United States[1]. Increased bacterial endotoxin (lipopolysaccharide or LPS) has been suggested to be involved in the pathogenesis of NAFLD. In adults with NAFLD, elevated circulating endotoxin level has been reported when measured at multiple fasting time points[2,3]. Moreover, endogenous antibodies against endotoxin are found to be increased in adults with biopsy-proven nonalcoholic steatohepatitis (NASH), suggesting chronic exposure[4]. However, studies in pediatric population remain scarce and it is less clear whether or not endotoxin is an important mediator of NAFLD in the early forms of the disease as seen in children. One study by Alisi et al. indicates increased endotoxin levels in children with NAFLD as compared to healthy weight controls[5], but endotoxin could also be associated with obesity *per se*[6,7], thus warranting further examination.

Dietary fructose has been proposed to play a part in the pathogenesis of NAFLD, and endotoxin is possibly involved in this pathway through its interaction with Toll-like receptor 4 (TLR4). Animal model studies have suggested that high-fructose regimen is associated with increased portal blood endotoxin level, and reduction of endotoxin using oral antibiotics can lead to improvement of hepatic steatosis and inflammation[8]. Furthermore, in mice chronically exposed to fructose as compared to water-fed controls, there is a significant increase in hepatic triglyceride accumulation, portal endotoxin levels, as well as hepatic expression of genes of the TLR4-dependent signaling cascade[9]. This

effect of fructose feeding in hepatic steatosis is blunted in TLR4-mutant mice although portal endotoxin concentration remains similar comparing to wide type controls, confirming that NAFLD induced by fructose could be mediated by endotoxin[10]. In spite of the growing body of evidence from pre-clinical studies, limited data from humans are available in regards to the relationship between fructose and endotoxin in NAFLD. In particular, adolescents are an important group to focus on in view of their estimated highest intake of fructose[11] and increased prevalence of NAFLD[1].

In the current study, we utilized stored samples from cohorts of two clinical studies and sought to: 1) further investigate whether increased endotoxin level would be associated with the incretion of hepatic fat independently of obesity in adolescents; 2) characterize the association between dietary fructose consumption and endotoxemia in adolescents with NAFLD.

Methods

Subjects and study design:

Data from two cohorts of clinical studies were included for analysis. Both studies were approved by the Emory University and Children's Healthcare of Atlanta IRB, and written informed consent (parental consent obtained for subjects <18 years) and assent (when applicable) were obtained for each subject prior to initiation of the study.

Cohort 1 was comprised of 43 Hispanic, obese (BMI z-score ≥ 95th percentile for age and gender) adolescents (aged 11-18 years), who had self-reported high consumption of sweet beverages (at least 3 servings of 12 fl oz bottles per day on average). Sweet beverages were defined as drinks sweetened with added sugars (eg, sodas, sweet tea, sport drinks, flavored drinks) or naturally sweet beverages (eg, 100% fruit juice), but did not include artificially-sweetened drinks. Because both Hispanics race[12] and high intake of sweet beverages[13] have been reported to be risk factors for hepatic steatosis, we were able to recruit a group of adolescents in this cohort who were likely to have increased risk of significant steatosis but without the prior diagnosis and treatment. Subjects were recruited from local pediatric clinics and from nearby community centers through flyers and presentations at community events. Exclusion criteria included known liver diseases; diabetes or fasting glucose > 126 mg/dl; renal insufficiency (creatinine > 2 mg/dl); any chronic disease requiring daily medication; acute illness within past 2 weeks prior to enrollment (defined by fever > 100.4ºF); and supplement and/or anti-oxidant therapy within 4 weeks prior to enrollment. All recruited participants underwent magnetic resonance spectroscopy (MRS) to quantify their hepatic fat. Subjects with hepatic fat > 5% were considered as having steatosis[14] (n = 32), and they were otherwise classified as obese controls (n =11) if MRS-documented hepatic fat < 5%. Next, a subgroup of adolescents with hepatic steatosis (n=24) participated in a 4-week calorie-matched, well-controlled beverage intervention trial. Briefly, subjects were randomly assigned to either study-provided fructose- (considered as fructose continuation) or glucose-containing beverages (considered as fructose reduction) and were instructed to drink 3 servings of 12 fl oz bottle each day for 4 weeks. These beverages contain 33 grams of sugar (standard

amount of sugar in a typical soda) in the form of either glucose or fructose, matched for color and flavoring (Power Brands, Beverly Hills, CA). Participants and investigators were blinded as to the contents of the drinks. No other sugar-containing beverages were allowed during the study period, and subjects were requested not to change their diet pattern and physical activity. Follow-up visits were scheduled at 2 and 4 weeks after the initiation of randomization. For each study visit, subjects were required to fast for at least 12 hours and fasting blood samples were collected for the laboratory measurements. MRS was repeated to monitor the change of hepatic fat content, and body weight of subjects was also recorded at each visit. Compliance was monitored through daily drink logs, return of empty beverage bottles at each study visit, and weekly phone calls from the study coordinator. A total of 21 adolescents successfully completed this study protocol but only 16 had endotoxin data available for analysis.

Cohort 2 included a small group of adolescents with biopsy-proven NAFLD and healthy controls. The study design and methods were previously described elsewhere[15]. Briefly, it was a 24-hour, crossover feeding study and subjects were randomized to either glucose or fructose beverages at visit 1 and the other sugar at visit 2. For each of two visits, they consumed a standardized meal consisting of 700 kcal and 78 g sugar the evening before the study and then fasted for 12 hours before the initial baseline measurements. On the next morning, subjects arrived at the inpatient research unit at 0700h and were given either high fructose or glucose (33% of total estimated daily calories) beverages along with a typical breakfast (0800h), lunch (1200h), and dinner (1600h). Standardized meals (containing 50% carbohydrates, 30% fat, and 20% protein) were prepared in the

metabolic kitchen, and typically included items such as scrambled eggs, a hamburger, tater tots, and green beans. Their blood samples were drawn at 0800h (baseline) before feeding, 1h after breakfast, and subsequently, every 2h until the following morning except at 0400h to allow the adolescents to sleep. All subjects (n = 8 for biopsy-proven NAFLD, and n = 7 for non-NAFLD controls) with non-hemolyzed baseline samples and sufficient postprandial samples available were included for the analysis of endotoxin. We had a focus on the fructose-beverage feeding day only in this preliminary work to compare the difference of postprandial endotoxin levels in response to fructose between NAFLD and non-NAFLD subjects.

Measurement of Hepatic fat:

Hepatic fat was assessed by MRS using our previously described methods[16]. Briefly, we used a rapid 15-sec acquisition technique obtained during a single breath hold. The sequence is constructed from five concatenated echoes using a fixed set of echo times (TE) (12, 24, 36, 48, and 72ms), with each echo having a repetition time (TR) = 3000ms, voxel = 3 x 3 x 3 cm3, 1024 points, and 1200 Hz bandwidth. The acquisition was repeated three times for reproducibility. Data were exported off-line for automatic processing with in-house software (Matlab, Mathworks, Natick, MA). Water and lipid magnitude spectra were analyzed by determining the area under the curve (AUC) corresponding to a user-defined frequency range surrounding the corresponding water/lipid peaks (water peak: 4.6ppm; lipid peak: 1.3, 2.0ppm). The integrated magnitude signals at each TE were fit to exponential T2 decay curves, whereby the

equilibrium signal (M0) and the relaxation rate (R2 = 1/T2) were determined by least-squares regression approximation. Using M0 for water and lipid, the T2-corrected hepatic lipid fraction was calculated from: % Hepatic Lipid = M0lipid / (M0lipid + M0water).

Laboratory measurement:

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) tests were performed by the Emory University Hospital clinical laboratory. Plasma glucose, insulin, and high sensitivity C-reactive protein (hs-CRP) were measured with immunoturbidometric methods (Sekisui Diagnostics, Exton, PA) using AU480 chemistry analyzer (Beckman Coulter), by the Emory Lipid Research Laboratory. Plasma tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and monocyte chemotattractant protein-1 (MCP-1) were determined with multi-analyte chemiluminescence detection using Luminex® xMap technology (Millipore Corporation, St. Louis, MO). Endotoxin levels were evaluated by colorimetric assay. Plasma samples were diluted 2-fold in pyrogen-free water, mixed, and heated at 75°C for 10 min to remove non-specific inhibitors of endotoxin. Samples were allowed to cool to room temperature before colorimetric assay using the limulus ameobocyte lysate (LAL) kit (Lonza Walkersville, MD). Standards and samples were incubated with LAL for 10 min at 37°C followed by 6-min incubation with colorimetric substrate. The reaction was stopped with 25% acetic acid, and the absorbance was read at 405 nm.

Insulin resistance index:

Insulin resistance was assessed by homeostasis model of assessment - insulin resistance (HOMA-IR), which was calculated by glucose (mg/dl) x insulin (μU/L)/405 at fasting state.

Statistical analyses:

Statistical analyses were performed using SAS 9.1. Results in tables were expressed as mean (SD) unless indicated otherwise. Statistical significance was considered as $p \leq 0.05$. Data were examined for normality and equal variance prior to any analyses. Independent two sample t-tests or alternatively Mann-Whitney tests (if not normally distributed) were used for comparison between subjects with and without NAFLD (steatosis). Paired t-tests were conducted to determine the significance of percent change in endotoxin levels at week 2 and 4, as compared to baseline. Spearman's tests were performed to examine the correlations of plasma endotoxin. In the feeding study, the 24-hour incremental area under the curve (IAUC) was calculated for endotoxin by using the trapezoidal method; and independent comparisons were performed for each single time point between NAFLD and non-NAFLD subjects.

Results

Hepatic steatosis and plasma endotoxin concentration (cohort 1):

Anthropometrics and laboratory parameters for the 32 adolescents with hepatic steatosis and the 11 obese controls are reported in **Table 1**. There were no differences in age, gender, body weight, and BMI z-score between the two groups. Adolescents with hepatic steatosis (> 5% by MRS) had significantly increased hepatic fat ($p < 0.001$), ALT ($p = 0.021$), AST ($p < 0.001$), fasting insulin ($p = 0.010$), and insulin resistance as assessed by HOMA-IR ($p = 0.013$) compared with obese control subjects. Plasma concentration of endotoxin in obese controls averaged at 1.22 ± 0.30 EU/ml (mean ± SD, ranging from 0.64 to 1.61 EU/ml); while in subjects with steatosis, mean endotoxin level increased to 1.54 ± 0.52 EU/ml (mean ± SD, ranging from 0.85 to 2.83 EU/ml) ($p = 0.019$) (**Figure 1**). The plasma endotoxin level was positively correlated with insulin ($r = 0.403$, $p = 0.008$); HOMA-IR ($r = 0.332$, $p = 0.013$), TNF-α ($r = 0.381$, $p = 0.029$), and MCP-1 ($r = 0.386$, $p = 0.022$). No significant association was observed between plasma endotoxin and ALT levels.

Acute endotoxin response to fructose feeding (cohort 2):

A group of 8 adolescents with histologically confirmed NAFLD and 7 healthy controls were included for endotoxin analysis and their baseline characteristics are summarized in **Table 2**. To examine the acute effect of fructose feeding on endotoxin level, we compared the postprandial (0-9 hrs) and persistently daylong (0-23 hrs) responses between NAFLD and non-NAFLD subjects by calculating the IAUC. We found that adolescents with NAFLD had increased 9h-IAUC with consumption of three consecutive high fructose meals when compared to non-NAFLD (mean ± SE: 6.85 ± 1.49 vs. 2.50 ±

0.87, p = 0.026) (**Figure 2**). The biggest differences were after breakfast and lunch and less difference was seen overnight, thus the 23h-IAUC comparison (mean ± SE: 15.11 ± 3.83 vs. 10.19 ± 4.23, p = 0.233) did not reach statistical significance between groups. When performing comparison at a given single time point, in adolescents with NAFLD, high fructose diet acutely increased their plasma endotoxin levels at 1h, 3h, and 5h, as compared to non-NAFLD controls.

4-week endotoxin response to fructose reduction (cohort 1):

Total of 16 adolescents with hepatic steatosis completed the 4-week randomized, calorie-matched intervention trial. Baseline characteristics of participants assigned to the two intervention arms are presented in **Table 3**. There were no significant differences in age, gender, weight, glycemic status, and lipid profile between the two groups. In the fructose continuation group, plasma endotoxin level continuously increased at both 2 weeks (p = 0.018) and 4 weeks (p = 0.088) after the initiation of intervention; while in adolescents who substituted glucose-only beverages for fructose beverages, their plasma endotoxin level was not further exacerbated within 4 weeks (**Figure 3**).

Discussion

With obesity being an important risk factor universally, NAFLD is now highly affecting both adults and children in the U.S. and presenting a major public health crisis. Endotoxemia has been postulated as a cofactor in the pathogenesis of NAFLD. This concept was strongly suggested in several studies by demonstrating increased leakage of

gut-derived endotoxin in adults with NAFLD[17,18]. Examining endotoxemia in pediatric NAFLD is important because children may have an early, possibly more aggressive form of the disease[19], and early disease may lead to lifelong morbidity and early mortality[20,21]. Alisi et al. previously reported a higher serum endotoxin concentration in children with NAFLD as compared to their healthy weight controls[5]. However, given the fact that increased endotoxin is associated with obesity *per se*[6,7], we further evaluated an entire group of overweight and obese adolescents (cohort 1) who had hepatic fat carefully quantified by MRS, allowing assessment of the relationship between endotoxin and hepatic steatosis irrespective of overweight and obesity. We found that adolescents with hepatic steatosis had an approximately 26% increase of plasma endotoxin comparing to their weight-matched controls, indicating hepatic steatosis in itself may be an independent factor.

Even a small increase of endotoxin could be clinically important. Previous work in healthy adults showed that administration of a low experimental level of endotoxin could cause an evident decrease in insulin sensitivity[22,23], and induce a rapid increase of circulating inflammatory markers including TNF-α, IL-6, hs-CRP, as well as MCP-1[22]. Accordingly, we reported that in our group of overweight and obese Hispanic-American adolescents (cohort 1), the elevation of their endotoxin levels significantly correlated with increased TNF-α, MCP-1, as well as insulin resistance as measured by HOMA-IR. Mechanism studies in mice have suggested that increased endotoxin can activate TLR4 to stimulate myeloid differentiation factor 88 (MyD88)[24]. This interaction of TLR4 and MyD88 triggers the downstream signaling cascade leading to the activation of the nuclear

factor κB (NF-κB) pathway, further releasing inflammatory cytokines such as TNF-α and IL-6[25]. Endotoxemia-induced increase of inflammatory cytokines such as TNF-α[26] can inhibit insulin receptor signaling and targets insulin receptor substrate proteins for degradation[27]. In addition, endotoxin is also known to markedly induce MCP-1 which can recruit chemokine CC motif receptor (CCR)-2-expressing monocytes, and thereby promotes insulin resistance[28] and hepatic inflammation[29].

Recent investigations indicate that an individual's diet may have a strong influence on endotoxin translocation. The westernized-style diet is characterized by the consumption of high amounts of saturated fat and simply sugar, especially fructose. Fructose, a sweetener widely used in beverages and many processed foods, has caught most attention because consumption of fructose-sweetened products has incrementally increased fivefold in the last century and doubled in the last thirty years[30]. Evidence from experiments in mice suggested that fructose ingestion was associated with marked loss of tight junction protein and intestinal bacterial dysbiosis, resulting in increased circulating endotoxin[8,9]. Recently, these findings were further extended in non-human primates, a more relevant model to human[31]. However, fructose-induced endotoxin to disease is less well understood in humans, especially in adolescent group. Feeding study is an essential way to study the response of endotoxin in various durations. In our study cohort 2, we examined endotoxin responses to three consecutive meals with fructose-sweetened beverages in adolescents with biopsy-proven NAFLD and healthy controls. Both NAFLD and non-NAFLD subjects had an acute elevation of postprandial endotoxin levels, but there was a significantly higher response observed in adolescents with NAFLD. This

increased susceptibility in NAFLD might be explained by increased gut permeability, disruption of intestinal tight junctions, and possible alterations in the microbiome as indicated in adults[17,18]. Alternatively, it is also could be due to the impaired Kupffer cell function. Normally, endotoxin released from the gut is cleared rapidly on first pass by Kupffer cells to prevent its escape into the systemic circulation. In NAFLD, this hepatic clearance may be disturbed and insufficient[32] and could be responsible for the increased level of circulating endotoxin.

There is a growing body of evidence linking high fructose intake to NAFLD, and endotoxin could be a possible mediator in this pathway. NAFLD patients have been found to have increased consumption of dietary fructose[3,33,34] and fructose is associated with severity of fibrosis in NAFLD patients[35]. The mechanisms through which fructose-induced endotoxin might contribute to NAFLD have been extensively studied but little data were previously available in the pediatric population. We thereby performed a calorie-matched, randomized, well-controlled beverage trial in adolescents with hepatic steatosis examining their endotoxin response to a 4-week continuation or reduction of fructose-sweetened beverages. We found that 4-week of fructose reduction through replacing with glucose-only study-provided beverages did not further exacerbate plasma endotoxin levels, while continuation of fructose beverages continuously promoted the elevation of endotoxin. An adult pilot study demonstrated that subjects with NAFLD who consumed 50% less fructose compared to baseline for a six month period had significantly lower levels of endotoxin as well as hepatic lipid contents[36]. However, causality could not be proven because the subjects also lost weight. In our trial, body

weights of the subjects remained stable from baseline to the end of the intervention. Although we did not observe an improvement in endotoxin levels with fructose reduction due to the limited study length, we have previously reported a significant improvement in insulin resistance and inflammatory status, and this could be the downstream effects of well-managed endotoxin levels. Putting our data together with the previous studies, it appears likely that when subjects are chronically exposed to a high fructose environment, endotoxemia and subsequent activation of inflammatory cytokines promote liver injury and play a detrimental role in NAFLD.

Our study has several important strengths. First, our study cohort 1 consisted entirely of 43 obese adolescents allowing us to isolate the effect of hepatic steatosis independently of body weight and examine its associations. Also, we utilized the state-of-the-art MRS methodology as the evaluation of hepatic steatosis to increase its accuracy. Finally, samples of our cohort 2 were from an inpatient feeding study, which helped to improve the compliance. We also controlled the meal the night before the day of the feeding study to limit the variations of endotoxin response.

Our preliminary work is also subject to some limitations. In study cohort 1, our subjects are Hispanic-American adolescents, thus our findings may not be generalizable to other racial and ethnic groups, particularly African-American children, who appear to be protected from progressing to significant hepatic steatosis. We did not see a correlation between plasma endotoxin and ALT, but this might in part due to the fact that only a few

subjects had elevated ALT in our cohort 1. In our feeding study (cohort 2), the sample size was small and future investigation with a larger group will be needed to better examine the fructose-induced endotoxin response in pediatric population. In the fructose reduction trial, 4-week study period was brief compared to the years that adolescents typically consume sweet beverages and a longer study would be helpful to better characterize the relationship between fructose restriction and potential endotoxin improvement in NAFLD.

In conclusion, we demonstrated that exposure to high fructose environment both acutely (24 hours) and chronically (4 weeks) induced an increase of circulating endotoxin in adolescents with NAFLD, and we also demonstrated correlations of endotoxin with markers of insulin resistance and inflammation. Fructose reduction would be a feasible side-effect-free strategy for patients with NAFLD to prevent disease progression. However, larger controlled trials will be necessary to prove the therapeutic and possibly preventive benefits of fructose reduction.

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The authors' responsibilities are as follows: Vos, designed research, supervised study, wrote parts of the manuscript, and has primary responsibility for final content; Jin, performed part of laboratory measurements, analyzed and interpreted the data, wrote parts of the manuscript; Willment, Song, and Mannerly, performed part of laboratory measurements; Patel, contribute to data analyses; Sun, performed and examined the statistical analyses; Kosters and McClain, assisted with data interpretation and contribute to the development of the manuscript.

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Table V-1. Anthropometrics and laboratory parameters of the 32 adolescents with hepatic steatosis and the 11 obese controls at fasting state - study cohort 1

Parameters, mean (SD)	**Obese controls (< 5% by MRS, n =11)**	**Subjects with steatosis (> 5% by MRS, n =32)**	**p-value**
Age, years	14.3 (1.85)	13.7 (2.65)	0.443
Male (n, %)	4 (36.4)	13 (40.6)	0.803
Weight (kg)	82.9 (21.5)	80.5 (14.9)	0.967
BMI z-score	2.00 (0.22)	2.17 (0.37)	0.129
ALT (U/L)	17.8 (7.60)	49.7 (89.5)	0.021
AST (U/L)	21.9 (3.99)	68.2 (180)	<0.001
Hepatic fat (%)	3.87 (0.62)	11.2 (5.27)	<0.001
Glucose (mg/dl)	92.4 (16.0)	93.4 (16.2)	0.978
Insulin (µU/L)	18.2 (6.69)	36.2 (30.5)	0.010
HOMA-IR	4.06 (1.31)	8.79 (9.13)	0.013
hs-CRP (mg/L)	3.17 (3.44)	4.98 (5.92)	0.278

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOMA-IR, homeostatic model assessment for insulin resistance, calculated as fasting Glucose (mg/dl) x insulin (µU/L)/405; hs-CRP, high sensitivity C-reactive protein

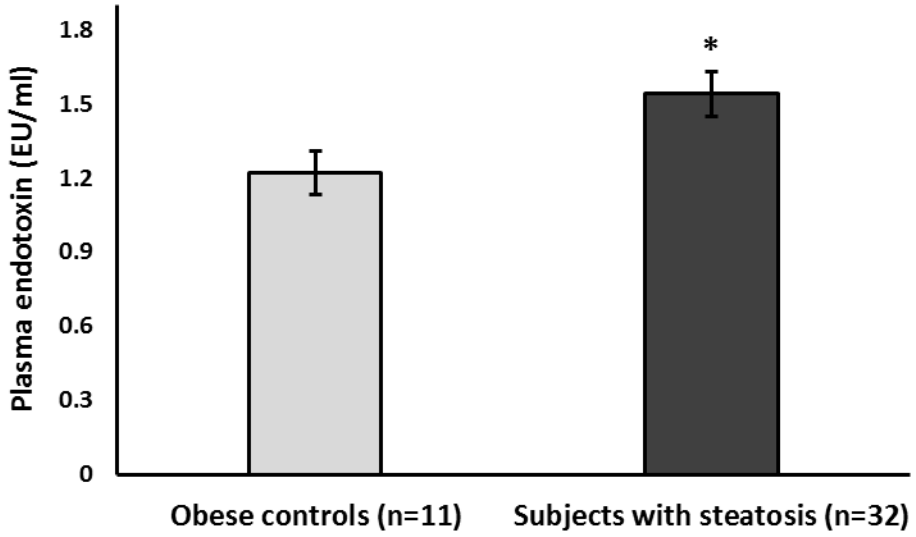

Figure V-1. Adolescents with hepatic steatosis (> 5% by MRS) had increased plasma endotoxin levels as compared to theirs obese controls (hepatic fat < 5% by MRS); * $p < 0.05$.

Table V-2. Baseline characteristics of the 8 adolescents with biopsy-proven NAFLD and the 7 healthy controls - study cohort 2

Parameters, mean (SD)	**non-NAFLD (n=7)**	**NAFLD (n=8)**	**p-value**
Age, years	13.7 (2.22)	13.0 (2.73)	0.315
Male, n(%)	5 (71.4)	8 (100)	0.104
BMI z-score	0.18 (0.65)	2.29 (0.38)	0.001
Hepatic fat, %	1.02 (1.18)	22.0 (6.16)	0.001
ALT (U/L)	14.6 (2.51)	130 (63.2)	0.001
AST (U/L)	23.4 (4.30)	79.6 (40.6)	0.001
Glucose (mg/dL)	99.6 (7.18)	95.9 (19.1)	0.487
Insulin (µU/L)	9.67 (12.4)	42.7 (27.7)	0.005
HOMA-IR	2.27 (2.70)	10.2 (6.99)	0.016
hs-CRP (mg/L)	0.25 (0.49)	2.95 (3.33)	0.004

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOMA-IR, homeostatic model assessment for insulin resistance, calculated as fasting Glucose (mg/dl) x insulin (µU/L)/405; hs-CRP, high sensitivity C-reactive protein

Table V-3. Baseline characteristics of participants enrolled in the 4-week beverage intervention trial – study cohort 1

Parameters, mean (SD)	**Fructose (n=8)**	**Glucose (n=8)**
Age (years)	14.6 (2.50)	13.3 (2.32)
Male, n (%)	3 (37.5)	4 (50.0)
Body weight (kg)	86.1 (13.3)	81.3 (15.9)
BMI z-score	2.32 (0.56)	2.01 (0.26)
Hepatic fat (%)	14.5 (5.73)	12.1 (4.82)
ALT (U/L)	35.1 (20.5)	31.3 (18.6)
AST (U/L)	32.1 (9.76)	32.9 (7.45)
Triglycerides (mmol/L)	161 (111)	175 (58.5)
Cholesterol (mmol/L)	166 (28.8)	170 (48.8)
LDL (mmol/L)	106 (32.5)	105 (38.6)
HDL (mmol/L)	45.1 (9.84)	45.0 (9.83)
FFA (mmol/L)	0.97 (0.24)	1.16 (0.51)
Glucose (mmol/L)	98.3 (15.4)	89.6 (28.6)
Insulin (pmol/L)	30.0 (13.7)	31.4 (30.5)
HOMA-IR (mmol/L·pmol/L)	7.17 (3.03)	7.23 (8.28)
hs-CRP (mg/L)	4.22 (3.03)	3.16 (2.54)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FFA, free fatty acid; HOMA-IR, homeostatic model assessment for insulin resistance index, calculated as fasting Glucose (mg/dl) x insulin (μU/L)/405; hs-CRP, high sensitivity C-reactive protein

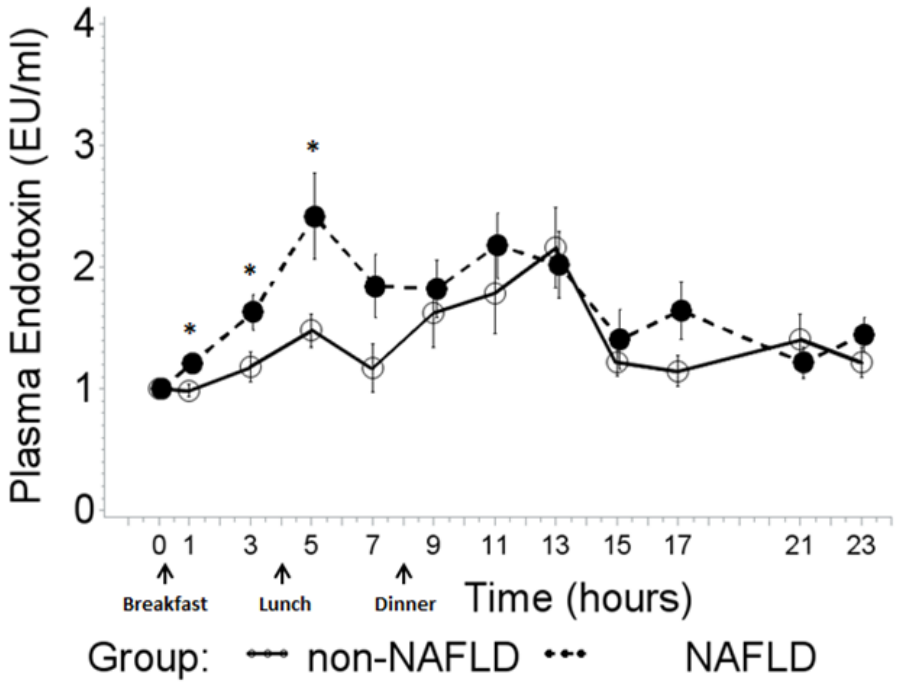

Figure V-2. Postprandial plasma endotoxin levels in response to fructose beverages given with breakfast, lunch and dinner. The solid line represents 7 children without NAFLD and the dashed line shows the response in 8 children with biopsy proven NAFLD. Baseline values were set as reference (1) and the following time points represent the ratio to baseline. * $p < 0.05$ when comparing NAFLD and non-NAFLD subjects at a given time point.

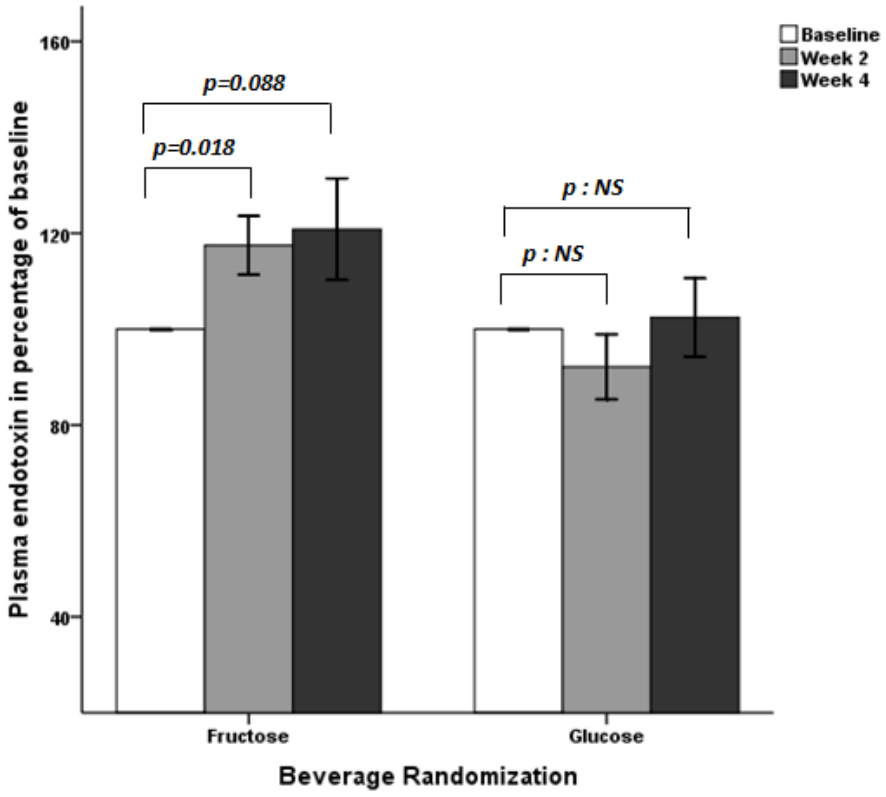

Figure V-3. Percentage change of plasma endotoxin level in adolescents with NAFLD after 2 and 4 weeks ingestion of fructose beverages or glucose-only beverages. Baseline values were set as reference (100%). Error bars stand for SE.

Chapter VI: Conclusions, Future Directions, and Public Health Implications

Conclusions

Currently, nonalcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease. NAFLD can progress to nonalcoholic steatohepatitis (NASH) and ultimately end-stage liver diseases that might need liver transplantation. More importantly, NAFLD has been reported to be a risk marker of cardiovascular disease (CVD) and might also a mediator involved in the pathogenesis of CVD[1,2]. Overconsumption of dietary fructose has been proposed as a culprit in the development and progression of NAFLD, and may be particularly associated with increased CVD risk as seen in NAFLD. By performing a 24-hour inpatient feeding study, we demonstrated that high-fructose-diet induced a postprandial elevation of plasma triglycerides (TG) and a rapid decline of high density lipoprotein (HDL) in adolescents with NAFLD. This fructose-induced high TG – low HDL dyslipidemic phenotype may be closely involved in the pathogenesis of early atherosclerosis in adolescents with NAFLD and predicts their future cardiovascular event[3].

Given the fact that fructose consumption represents a significant source of daily calories in adolescents[4], habitual reduction of dietary fructose may be particularly important in regards to reduce cardiovascular risk factors in pediatric NAFLD. Therefore, we conducted a 4-week calorie-matched, randomized, well-controlled fructose reduction in adolescents with NAFLD. We observed a significant improvement in their

cardiometabolic profiles including increased insulin sensitivity, reduced inflammation, and decreased low density lipoprotein (LDL) oxidation. Although our findings are very promising, the duration of this intervention is probably not long enough to detect its effect on hepatic steatosis and plasma lipids such as TG and HDL. A longer study would be helpful to confirm the persistence of these improvements observed in our current study, and to determine if a longer reduction would also improve liver findings and lipid profiles.

A number of mechanisms have been proposed that links fructose to NAFLD, although the role of fructose in pediatric NAFLD remains not fully elucidated. First, fructose stimulates de novo lipogenesis that contributes to increased supply of triglyceride in the liver and hepatic fat accumulation. Concomitantly, fructose induces increased visceral adiposity that actively produces inflammatory cytokines and promotes insulin resistance. Insulin resistance, nearly universally found in NAFLD, is associated with increased lipolysis and results in excess free fatty acid (FFA) flux to the liver. On the one hand, this expanded pool of fatty acid alters its metabolism through promoting hepatic *de novo* lipogenesis, oxidation in mitochondria, and esterification of triglycerides; and on the other hand, this overload of fatty acid in the liver exceeds its oxidative and very low density lipoprotein (VLDL) secretory capacity, thus further increasing the production of reactive oxygen species (ROS) and inflammatory cytokines to create a vicious cycle. In patients with NAFLD, they already have multiple defects in lipid metabolism, thus they may present less tolerance to fructose. In another word, fructose may exacerbate existing metabolic abnormalities in NAFLD and further triggers increased susceptibility to cardiovascular lesions.

We presented our preliminary data showing that fructose-induced endotoxin could be a mediator involved in pediatric NAFLD. We demonstrated that fructose acutely (24 hours) induced the postprandial elevation of plasma endotoxin, and chronic exposure (4 weeks) to high fructose environment further exacerbated this elevated level of endotoxin in adolescents with NAFLD. We also demonstrated correlations of endotoxin with markers of insulin resistance and inflammation. Thus, fructose reduction could be a side-effect-free strategy for patients with NAFLD to manage endotoxemia and consequently their increased CVD risk as well.

Future directions

In the current study, we reported the improvement in several CVD-related markers with a 4-week fructose reduction in adolescents with NAFLD. In the future work, non-invasive vascular measurements of early atherosclerosis will be needed to directly evaluate the long-term benefit of fructose restriction on cardiovascular endpoint. Of note, we designed our fructose feeding study with a high dose of fructose (33% of total calories) that is not likely to be consumed on a daily basis by most children. However, in the highest consumers of added sugars among children, 33% of the diet often comes from sugars[5]. So far, the "safe" upper limit of fructose intake for children has not been well established. Future efforts are necessary to help provide the guideline of fructose consumption for the purpose of educating parents and other caregivers to limit their children's experience of soft drinks and other sweets rich in fructose.

Public Health Implications

In spite of the increased prevalence of NAFLD, well-established treatment options are still not currently available. Weight loss is regarded as the first-line management, primarily through diet modification and exercise. Our 4-week fructose reduction demonstrated an improved cardiometabolic profile in adolescents with NAFLD, suggesting that fructose is a feasible and inexpensive dietary intervention for NAFLD and it could be considered as an adjunct to current therapies.

More importantly, habitual reduction of fructose for adolescents is needed to prevent the development and progression of NAFLD, as well as the future cardiovascular event. From the standpoint of public health, fructose restriction could be achieved through 1) well-designed educational programs to instruct parents and other caregivers to limit their children's intake of high-fructose-containing products such as soft drinks; 2) the replacement of sodas and other soft drinks with healthier beverage choices (eg, plain water) from vendor machines and cafeterias at schools; 3) the removal of sugar-sweetened beverages from the Supplemental Nutritional Assistance Program (SNAP); and 4) the increase of tax on soft drinks to possibly limit future purchases.

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