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Jeffrey Holzberg Date

The Role of Plasminogen Activator Inhibitor-1 in Hepatic Steatosis, Dyslipidemia, Insulin Resistance and Fructose Consumption in Children with Fatty Liver Disease

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The Role of Plasminogen Activator Inhibitor-1 in Hepatic Steatosis, Dyslipidemia, Insulin Resistance and Fructose Consumption in Children with Fatty Liver Disease

By

Jeffrey Holzberg
B.S., Tufts University, 2007

Advisor: Miriam Vos, MD, MSPH

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

The Role of Plasminogen Activator Inhibitor-1 in Hepatic Steatosis, Dyslipidemia, Insulin Resistance and Fructose Consumption in Children with Fatty Liver Disease

By Jeffrey Holzberg

Background: The increased morbidity and mortality in patients with nonalcoholic fatty liver disease (NAFLD) is in large part due to complications of cardiovascular disease (CVD). 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. Plasminogen activator inhibitor-1 (PAI-1) has been proposed as a link between fructose consumption, NAFLD and CVD. Thus, we examined PAI-1 in relation to fructose consumption and the development of hepatic steatosis and insulin resistance among overweight and obese children.

Methods: Our study consisted of two parts. In the first, 39 overweight and obese Hispanic children underwent comprehensive anthropometric and metabolic assessment as well as magnetic resonance spectroscopy for hepatic fat calculation. In the second part, 24 children from cohort 1 who had hepatic steatosis >5% on imaging and that were regular consumers of sugary beverages were enrolled in a 4-week randomized-controlled, double blind intervention study using calorically matched fructose and glucose beverages and evaluated at 0, 2 and 4 weeks.

Results: PAI-1 was found to be closely correlated with severity of hepatic steatosis independent of BMI, visceral fat, and insulin resistance ($p < 0.001$). PAI-1 did not significantly correlate with insulin resistance after controlling for BMI. Further, plasma PAI-1 did not significantly change over the four week period among those consuming fructose supplement or glucose supplement, or comparing the change between the two groups.

Conclusion: These findings suggest that PAI-1 most closely relates to hepatic steatosis in early stages of fatty liver development in children, independent of other risk factors, and that hepatic steatosis may mediate PAI-1's association with insulin resistance. PAI-1 offers the potential to be elevated among high-fructose beverage consumers but further studies are needed to observe this trend. Finally, PAI-1 may serve as a biomarker for NAFLD development early in the course of its disease and possibly as a target to prevent NAFLD progression into NASH or CVD complications.

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I would like to also acknowledge that many of these results are in the process of being published in two studies. First is titled "Hepatic Steatosis is linked to Plasminogen Activator Inhibitor-1 Independent of Body Weight in Pediatric Nonalcoholic Fatty Liver Disease" with authorship: J Holzberg, R Jin, Y Mannery, NA Le, T Ziegler, E Blunt, C McClain, G Arteel and M Vos. The second is "Dietary Fructose Reduction Improves Markers of Cardiovascular Disease Risk in Adolescents" with authorship: R Jin, J Welsh, NA Le, J Holzberg, P Sharma, D Martin and M Vos.

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INTRODUCTION:

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide in adults and children.[1,2] The disease encompasses a spectrum of pathological conditions ranging from simple steatosis (fat infiltration of >5% of hepatocytes in the absence of excessive alcohol intake) to nonalcoholic steatohepatitis (NASH), fibrosis and liver cirrhosis.[3] In children, NAFLD is particularly concerning because of its progressive nature and lifelong liver- and cardiac-related morbidity and mortality.[4,5] In all age groups, NAFLD is closely intertwined with features of metabolic syndrome, most notably obesity, insulin resistance and dyslipidemia,[6-11] and independently predicts cardiovascular disease (CVD) events.[12-16] The exact mechanistic pathways that underlie development of hepatic steatosis remain poorly understood, as does the link between NAFLD and CVD.[17] The fibrinolytic system, which is linked with NAFLD,[18,19] insulin resistance,[20] and atherosclerosis,[21] is likely involved in the pathogenesis of NAFLD and may help to investigate possible targeted interventions.

Plasminogen activator inhibitor-1 (PAI-1) is an acute-phase protein produced primarily by hepatocytes and adipocytes. It inhibits the serine proteases tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), and thereby attenuates fibrinolysis and inhibits the activation of hepatocyte growth factor (HGF) (Figure 1).[22-24] PAI-1 is best known for its role in vascular thrombosis and atherosclerosis in patients with CVD[25,26] and has been known for years to be elevated in obesity, dyslipidemia and insulin resistance.[20,27,28] More recently, some studies have shown PAI-1 to be associated with hepatic steatosis and NAFLD development.[18,29,30] This association is independent of BMI and sex, and implicates PAI-1 as a potential biomarker or critical

step in the mechanism of NAFLD progression. However, there are mixed results between studies in part because of utilization of less precise fat measurement like ultrasound[31-33] and lack of characterization of important confounders like visceral adiposity and insulin resistance.[31,33-35] Additionally, virtually no studies have been done in children, a crucial period in NAFLD development.

In view of the high prevalence of NAFLD among adolescents[36] and the strong association between NAFLD and CVD, it is also essential to explore early prevention strategies through diet and lifestyle modification. Fructose is a widely used sweetener in beverages and many processed food and its consumption has increased dramatically over the last 40 years.[37] Recent data have shown that fructose intake represents ~12% of daily calories consumed by U.S. adolescents, primarily from sugary beverages.[38] In both animal and short-term human feeding studies, fructose has been found to increase hepatic fat accumulation through insulin resistance, lipogenesis, and plasma hypertriglyceridemia.[39-41] Fructose intake has also been suggested in numerous studies as part of the mechanistic pathway that elevates plasma PAI-1.[30,42-44] However, direct evidence showing the benefits of fructose restriction on PAI-1, 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.[36,38]

STUDY AIMS:

Thus, the present study was done in children with fatty liver disease to assess the relationship between PAI-1 and hepatic steatosis, insulin resistance and fructose consumption; thus clarifying the role of PAI-1 in NAFLD development. This thesis has two study aims with diagrammatic explanation in Figure 2. Our first aim is to examine the association between PAI-1 and various metabolic impairments in overweight and obese Hispanic children. The primary outcome of interest is hepatic steatosis, while secondary outcomes of interest include insulin resistance and dyslipidemia. We hypothesized that an elevated level of PAI-1 is associated with an elevated level of hepatic steatosis, insulin resistance and dyslipidemia independent of weight.

Our second aim is to examine the effect of switching to consumption of beverages high in glucose as opposed to fructose on the concentration of plasma PAI-1 after 14 and 28 days among overweight and obese Hispanic children with NAFLD who are known to be high fructose consumers. We hypothesized that PAI-1 levels will be lower in the group of children who switched from fructose to glucose compared to the group that continued to consume fructose.

METHODS:

Study Population

This sample consisted of Hispanic overweight and obese children prospectively recruited from pediatric clinics at Emory Children's Center and from nearby community centers through flyers and presentations at community events during the summer of 2012 and then studied in the Atlanta Clinical and Translational Science Institute (ACTSI) Clinical Research sites at Children's Healthcare of Atlanta (CHOA) and Emory University Hospital.

Eligibility criteria included children aged 11-18 years, self-identified as Hispanic; body mass index (BMI) ≥ 85th percentile for age and gender; and average self-reported consumption of at least 3 sugar-containing drinks per day. We chose to study obese Hispanic adolescents because they are at particularly high risk of metabolic syndrome and hepatic steatosis.[11,45] Sugar containing beverage consumption was an inclusion criteria in order to increase the likelihood of finding adolescents with significant steatosis.[46,47] These beverages were defined as sodas, flavored drinks, 100% juice, and other beverages containing primarily fructose and water. Although 100% juice is not typically included in assessment for sugar consumption, we included them because the fructose content of juice is high and little evidence exists to suggest that it would not have the same effects as fructose from soda.

Exclusion criteria included known liver diseases; diabetes or fasting glucose > 126 mg/dL; renal insufficiency (creatinine > 2 mg/dl); any chronic diseases required daily medication; acute illness within past 2 weeks prior to enrollment (defined by fever > 100.4ºF); and supplement or anti-oxidant therapy within past 4 weeks before enrollment.

39 adolescents who met the eligibility criteria were recruited for the Aim 1 Sample of this cross-sectional study. These subjects underwent an anthropometric assessment and MRS procedure for the determination of hepatic fat content and a blood sample collection in the fasting state.

The second aim of this study would examine fructose as a possible cause of elevated PAI-1. Aim 2 was a 28 day, double-blinded, parallel armed randomized controlled trial of a subset of the 39 overweight and obese Hispanic adolescents enrolled in Aim1 with MRI-confirmed NAFLD (n=27) (Figure 3). Of these 27 adolescents, 24 agreed to participate in our one-month cohort study. These 24 participants now in Sample 2 were randomized to receive 3 servings of 12 ounces per day of fructose-sweetened beverages or reduced-sugar sweetened beverages (glucose-based) over a 28-day period. Follow-up visits were scheduled at 14 and 28 days after the initiation of randomization.

After randomization, one subject with extremely elevated ALT was excluded and two subjects dropped out after 2 weeks. All 3 were in the fructose arm and all 3 were male. Therefore, a total of 21 subjects successfully completed the beverage intervention (Figure 3). Recruitment was originally set at 20 subjects per beverage arm to allow additional within-group comparisons; however the study was halted at 24 subjects after a policy change that no longer allowed our pediatric subjects to obtain MRS at the adult center. By using a 3% mean change of hepatic fat and standard deviation, the power analysis for the project had estimated 6 subjects per beverage group to achieve >90% power, although this was done for lipid data rather than PAI-1.

During the 4 week study, participants were instructed to drink 3 servings (12 oz bottles) of study-provided beverages each day. The study beverages contained 33 grams

of sugar in the form of either glucose or fructose and were matched for color and flavoring (Power Brands, Beverly Hills, CA). This is similar to the energy supplied from three 12 oz sodas per day (typical soda has 33 grams of sucrose or high fructose corn syrup) and ~26% of the energy in a typical 2000 calorie diet. 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 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 participation in the study.

For all subjects, written informed consent was obtained from a parent or guardian and written assent was obtained from all children 11 years and older before participation. Demographic data were obtained from the parent. The protocols were approved by the Institutional Review Boards for Human Subject Research for Emory University (Atlanta, GA) and CHOA.

Variables. The following variables were measured for a total 39 subjects.

Anthropometric Measurements. Body weight, height and blood pressure were measured with the subject wearing light clothing with shoes removed. BMI was calculated as weight in kilograms divided by height in meters squared. BMI z-score/percentile was

determined according to age and gender, based on data from the Centers for Disease Control and Prevention.[48]

Laboratory Assay Measures. Baseline blood samples were drawn at 8 AM following a 12 hour overnight fast. Samples were collected into EDTA-coated tubes and plasma was separated immediately. Plasma samples were protected from light and transported in ice pack to the laboratory for further processing (within 4 hours). Samples were analyzed for aspartate aminotransferase (AST; U/L), alanine aminotransferase (ALT; U/L), gamma-glutamyl transpeptidase (GGT; U/L), and fasting insulin level (μU/ml). Plasma concentrations of AST and ALT were measured by routine enzymatic methods in the hospital clinical laboratory. Plasma cytokines, adipokines, chemokines, and pro-fibrotic markers were measured using multi-analyte chemiluminescent detection using Luminex® xMap technology (Millipore Corporation, St. Louis, MO USA). Specifically, adiponectin, resistin, tumor necrosis factor-α (TNF-α), PAI-1, C-reactive protein (CRP), were determined. Plasma insulin concentration was assessed using immunoturbidometric methods (Sekisui Diagnostics, Exton, PA).

All lipid measurements were performed by the Emory Lipid Research Laboratory at the Atlanta Veterans Affairs Medical Center using AU480 chemistry analyzer (Beckman Coulter). Total cholesterol and triglycerides (TG) were measured by enzymatic methods using reagents from Beckman (Beckman Diagnostics, Fullerton, CA). Low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were measured by homogeneous enzymatic assays (Sekisui Diagnostics, Exton, PA). Free fatty acids (FFA) were obtained by colorimetric methods (Sekisui Diagnostics, Exton, PA).

Insulin Resistance Measures. Two methods were used to evaluate insulin resistance. The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)[49] was calculated with the following formula: [Fasting plasma glucose (mmol/L) x fasting serum insulin (mU/mL)] / 22.5. Adipose tissue insulin resistance (Adipo-IR)[50,51] was calculated with the following formula: [fasting nonesterified fatty acids (mmol/L) x fasting insulin (pmol/L)].

Hepatic and Visceral Fat Measures. Hepatic steatosis was assessed by magnetic resonance spectroscopy (MRS) using our previously described methods.[52-54] 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 cumulative TE of 15,000 ms, with each echo having a TR=3000ms, voxel=3x3x3cm3, 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 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 approximation. Using M0 for water and lipid, the T2-corrected hepatic lipid fraction was calculated from: %Hepatic Lipid = M0lipid / (M0lipid + M0water). The calculated percentage of steatosis was then grouped depending on whether the steatosis was normal (< 5%), low (5-10%), or high (> 10%). Visceral adiposity was

measured using the seven scans taken in the abdomen region by MRI using previously-described methods.[55]

Statistical Analysis

For aim 1, descriptive analysis was done by dividing the study participants into three groups based upon quantification of hepatic fat (normal, low, and high). Group comparisons of means of the study variables were tested along this hepatic fat spectrum using the Kruskal-Wallis test, the nonparametric equivalent of the one-way analysis of variance (ANOVA) with Dunn's posttest, since most variables were not normally distributed. A significant test indicated that at least two of the groups were significantly different, in which case the Mann-Whitney U test was run to identify the groups of difference. Data are expressed as mean ± standard error with statistical significance considered as a p-value ≤ 0.05.

Linear regression was used to assess the predictive associations of the log-transformed PAI-1 with various independent continuous variables. This included hepatic fat, lipid measurements (TG, cholesterol, LDL, ApoB), and measures of insulin resistance (HOMA-IR, Adipo-IR, insulin). Since the distribution of PAI-1 as the outcome of interest was skewed (normality was examined with Komogorov Smirnov's test, skewness and kurtosis, table in appendix), it was log-transformed for the assumption of normality. The exact role played by PAI-1 has been historically been questioned because of its correlation with third factors that could potentially explain its association with atherosclerotic disease (such as diabetes mellitus, hypertension, obesity, dyslipidemia).[56] Therefore, regression models tested independence from these third variables, including

BMI, visceral adipose tissue (VAT), insulin resistance (as HOMA-IR) (Figure 4). Hepatic steatosis was controlled for when we tested it as a mediator between PAI-1 and other variables, such as lipids. BMI was of particular interest due to close correlation with PAI-1 in our study (Spearman's rho = 0.42, p = 0.0075) and prior studies.[20,57,58] Age and sex were not included in the model because of the limited sample size. Ordinal independent variables were included in the model as continuous variables. Spearman's correlations were also used to compare associations between continuous variables when appropriate.

For the descriptive analysis of aim 2, as the subjects were randomized, means and standard error are presented. For further analysis, treating PAI-1 as the dependent variable, a repeated measures model with beverage selection (fructose or glucose) and time as the independent variables factors were fitted. The interaction between beverage and time was also included as an independent variable. Individual was held constant in the model since the repeated measurements were among the same individual. Finally, to assess the change in PAI-1 continuously from baseline to 14 days (timepoint 1) and to 28 days (timepoint 2) between beverage groups, comparisons were made using a repeated measures ANOVA.

RESULTS:

Baseline Characteristics of Participants. Demographic characteristics and baseline variables for aim 1 and 2 are presented in Table 1 and Table 2 respectively. Cohort 1 included 39 Hispanic obese children (BMI z-score, mean ± SE: 2.09 ± 0.06 ng/mL) who self-reported high consumption of sugary beverages. In this group, 12 (30.8%) had normal hepatic fat (NHF, ≤ 5%), 14 (35.9%) had low hepatic fat (LHF, 5-10%), and 13 (33.3%) had high hepatic fat (HHF, > 10%). In cohort 2, 9 adolescents completed the study in the fructose group, as did 12 within the glucose group. All had the same range from 11-18 years old and nearly the same BMI z-score.

Aim 1

PAI-1 and Hepatic Fat. PAI-1 was positively associated with hepatic fat, increasing from 8.78 ± 1.17 ng/mL in the NHF group to 11.6 ± 0.99 ng/mL in the LHF group and 20.8 ± 2.26 in the HHF group ($p < 0.001$ by Kruskal-Wallis' test) (Figure 5). There was a significant difference between the NHF (0-5% fat) and LHF (5-10%) hepatic fat groups ($p = 0.04$), and LHF and HHF (>10%) groups ($p = 0.002$). Linear regression was used to further assess the relationship with the log of PAI-1 while controlling for confounding variables. Unadjusted analysis showed that hepatic fat was strongly associated with the log of PAI-1 ($\beta = 0.06$, $p < 0.0001$) (Table 3). Correcting for the child's BMI z-score, hepatic fat was found to have a strong association with the log of PAI-1 ($\beta = 0.056$, $p = 0.0005$) (Table 4). This association maintained significance even after controlling for insulin resistance instead (measured as HOMA-IR) (Table 5) or visceral adiposity (VAT) (Table 6) ($\beta = 0.057$, $p = 0.0004$; $\beta = 0.08$, $p = 0.001$; respectively).

PAI-1 and Dyslipidemia. We found that the log of plasma PAI-1 levels were closely associated with the plasma concentration of triglycerides ($p = 0.001$) and free fatty acids ($p = 0.007$) in the unadjusted analysis (Table 3). This remained true after controlling for BMI z-score ($p = 00009, 0.02$, respectively) (Table 4). Both triglycerides and free fatty acids maintained significance with the log of PAI-1 even after controlling for insulin resistance (HOMA-IR) (Table 5) ($p = 0.005$) or VAT (Table 6) ($p = 0.001$). Both associations with the log of PAI-1 lost significance after controlling for hepatic steatosis (Table 7). Other lipid measurements in the plasma were not significantly associated with the log of PAI-1, including cholesterol and LDL, in the unadjusted analysis or after controlling for BMI z-score (Table 3, 4).

PAI-1 and Insulin Resistance. Plasma the log of PAI-1 did not significantly associate with either HOMA-IR or Adipo-IR after controlling for BMI z-score (Table 4). Similarly, plasma insulin levels were positively associated with the log of PAI-1 levels ($p = 0.02$) but lost significance after controlling for BMI z-score. Neither measure of insulin resistance was found to significantly associate with the log of PAI-1 after controlling for hepatic steatosis (Table 7).

Aim 2

PAI-1 by Beverage Group. Plasma PAI-1 did not significantly change over the four week period among those consuming fructose supplement or glucose supplement (Table 8). A trend was present among the fructose and glucose groups (Figure 6), such that levels increased within the fructose group and decreased within the glucose group. Examination of the changes in PAI-1 on an individual level (in the fructose (Figure 7) and glucose

group (Figure 8)) demonstrated significant variability and a few extreme cases in the change in PAI-1 over 4 weeks.

PAI-1 by Beverage Group and Time. Repeated measures ANOVA with interaction did not demonstrate significance in the change of PAI-1 comparing fructose to glucose groups over the 1 month period (Table 8). A mixed model ANOVA regression was also performed to look for significant changes in PAI-1 between timepoints and beverage groups (Table 9). At baseline, there was no significant difference in PAI-1 between groups. Among glucose consumers, PAI-1 was shown to go decrease from baseline to 14 days (β = -1.45 ng/mL/14d) and from baseline to 28 days (β = -2.19 ng/mL/28d) but neither of these reached significance. Among fructose consumers, PAI-1 increased from baseline to 14 days (β = 3.35 ng/mL/14d), although the difference was not significant. Among fructose consumers, PAI-1 slightly decreased (β = -0.41 ng/mL/28d) from baseline to 28 days, but this difference was not significant. The change between the fructose and glucose supplement groups was not significant when compared from baseline to 14 days or from baseline to 28 days.

DISCUSSION:

The aim of this study was to investigate the role of PAI-1 in pediatric NAFLD by analyzing its associations with MRI-confirmed hepatic steatosis, insulin resistance, dyslipidemia and fructose consumption in a sample of Hispanic children that were high fructose consumers. PAI-1 was found to be closely correlated with severity of hepatic steatosis in children with NAFLD independently of BMI, visceral fat, and insulin resistance. There was no significant change in PAI-1 over a one month period among those who switched from fructose to glucose compared to those who kept drinking fructose supplement. These results support the involvement of PAI-1 in the pathogenesis of steatosis in children with NAFLD through its role in the buildup of hepatic fat irrespective of weight, visceral fat and insulin resistance, but do not support glucose substitution of fructose in beverages as an intervention to reduce PAI-1. Nonetheless, this study was not powered to detect smaller changes in PAI-1 levels that may have been caused by glucose substitution in sugary drinks.

The increased morbidity and mortality that is found in patients with NAFLD is mainly due to complications of CVD.[59-61] Although CVD events tend to occur in middle to late-aged adults, pathologic studies have shown that the subclinical atherosclerotic process begins in childhood[12,62-64] probably because of the dyslipidemia, insulin resistance and pro-inflammatory state seen in patients with NAFLD.[15,65-67] Some studies have sought to use PAI-1 to explain this link between NAFLD and CVD.[32] Similarly with NAFLD, PAI-1 has been linked to elevated CVD risk through increased numbers of atherosclerotic plaques, buildup of triglycerides and LDL, and contributions to a pro-inflammatory state.[68-70] Thus, it is important to clarify the relationship of PAI-1 with

NAFLD to understand its contribution to liver and cardiovascular pathology. It is equally important to examine modifiable influences such as fructose consumption that contribute to this increased cardiovascular risk in NAFLD.

AIM 1

The first conclusion of our study is that PAI-1 has a strong and independent association with hepatic steatosis. Studies that have previously linked PAI-1 to CVD have not accounted for the progression of hepatic steatosis in NAFLD development.[32,69] The association between PAI-1 and hepatic steatosis in our study is independent of the weight of the child or visceral adiposity, which are known to be strong predictors of the metabolic syndrome, NAFLD, and inflammatory biomarkers like adiponectin.[32,71] This finding suggests a possible role for PAI-1 as a direct biomarker and possible target for intervention in early development of hepatic steatosis. Our results suggest that plasma PAI-1 levels relate more to liver steatosis than fat accumulation in the extremities or abdominal cavity. This finding in children has not previously been reported in the literature but is consistent with reports in mice and adult humans. Alessi et al. showed that PAI-1 levels in adults are more closely related to liver steatosis than to visceral adiposity, although it is unclear how many cases of fatty liver were alcohol-induced.[34] In the same study, they showed that in genetically obese mice that develop a fatty liver early in life, PAI-1 expression was found to be significantly higher in the liver than in adipose tissue.[34] This is also consistent with studies in children that have shown an association between PAI-1 and hepatic steatosis but did not control for weight.[35,72]

Hepatic steatosis results from increased hepatic uptake of free fatty acids derived from hydrolysis of adipose-tissue triglycerides, dietary chylomicrons and hepatic

lipogenesis.[32,73,74] Our findings of a strong association between PAI-1 and triglycerides and free fatty acids help explain PAI-1's association with hepatic steatosis. These two associations remain significant even if controlling for VAT or HOMA-IR but lose significance when controlling for hepatic steatosis, strengthening the explanation of triglycerides and free fatty acids as the lipid content in the liver increases, thus having a significant relationship with PAI-1. From linear regression, we can deduce that an increase in hepatic steatosis by 1% is associated with an increase in PAI-1 by 5.6% (β of 0.056). Considering the 5% cutoff for diagnosing simple hepatic steatosis in NAFLD, the 1% increase in hepatic fat found to be associated with a 5.6% increase in PAI-1 is quite significant.

Our findings also suggest that hepatic steatosis may explain the association between PAI-1 and insulin resistance, which has been shown to be pathogenic in progression to steatohepatitis and CVD morbidity and mortality.[32,67,75] Insulin resistance has been associated with PAI-1 in previous studies, with some suggesting that hepatic steatosis actually mediates this relationship, initially thought to be mediated by adipose tissue.[34] The current study is the first to show this relationship in children. In a study by Ardigo et al., hyperinsulinemia is only associated with increased plasma PAI-1 in adults where there is also high liver fat content.[31] The results from our study show that PAI-1 is significantly associated with both HOMA-IR and Adipo-IR until the model controlled for hepatic steatosis, at which point significance was lost. This means that hepatic steatosis could be a common cause of both PAI-1 and insulin resistance, thus serving as a confounder with no true association existing between them. More likely, and consistent with prior studies,[31,34] hepatic steatosis exists in the causal pathway between PAI-1 and

insulin resistance, such that the association is lost when hepatic steatosis is held constant. Thus, the presence of hepatic steatosis could worsen the risk for insulin resistance in an overweight or obese child.

AIM 2

We also theorized that fructose reduction for 4 weeks would improve PAI-1 values. Hepatic fat has been shown by others to increase after soda consumption over 6 months.[76] 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.[77] In our study, neither group had a change in hepatic steatosis levels. While this disproved our hypothesis, our study was designed to be eucaloric. In other studies, the fructose reduction associated with loss of hepatic fat in adults was in the setting of weight loss; 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, there may not have been sufficient time to develop these downstream conditions from a change in fructose consumption. Perhaps a longer study would have resulted in changes in the liver. Given the proposed relationship between PAI-1 and steatosis, it is consistent that PAI-1 also did not change in either group.

Aims 1 & 2

Our study has several limitations. First, the cross-sectional model precludes assessment of causal or temporal relationships between PAI-1, hepatic steatosis, insulin resistance and inflammation. Additionally, visceral fat was not quantified directly but instead was inferred from body mass index. However, body mass index has been used

and accepted in prior studies as a measurement for visceral fat.[31,34] Third, as outlined before, the sample size in our study may have limited the significance of some associations. Since this was a pilot study, and one of the first looking at the association between PAI-1 and hepatic steatosis in children, there may not have been significant power to detect small changes. Finally, the sample was made up of overweight and obese Hispanic children, thereby limiting the generalizability of our results to other ethnic groups. For our second aim, we did not control the entire diet in our study subjects over the intervention period. This is not practical in children and would likely be less applicable to health recommendations. Compliance with supplement consumption was also difficult to control in this study, especially considering that the glucose supplement tasted objectively less sweet than the fructose supplement. This could limit the desire for the children to drink the glucose vs. fructose supplement.

Our study also has a number of strengths, including the use of MRI/MRS to quantify hepatic fat, the gold standard. This is also one of the first studies to look at NAFLD and PAI-1 in children, and the first study to take into account insulin resistance, and VAT in children. Our second aim was also a calorically-matched, double-blind, randomized controlled study comparing glucose to fructose beverages, a very strong format for an intervention study that is aimed to specifically assess the specific effects of fructose replacement with glucose on PAI-1.

Additional studies are needed to assess interventions that may reduce plasma PAI-1 concentrations and hepatic steatosis in children, including larger RCT's that specifically evaluate the potential role of fructose as a causal factor. Some studies have also suggested that angiotensin receptor blockers appropriately target PAI-1 and serve as a

potential treatment for NAFLD;[78,79] this is also being evaluated in an ongoing clinical trial by our group.

In conclusion, our results show that PAI-1 has a strong association with hepatic steatosis in children with NAFLD independent of visceral adiposity and BMI, likely due to the buildup of triglycerides and free fatty acids in the liver. Our study also demonstrates that the replacement of fructose with glucose for a 4 week period in adolescents with hepatic steatosis does not significantly reduce levels of PAI-1.

Table 1. Demographic and Metabolic Characteristics of Cohort 1

Parameters, mean (SE)	**NHF (N=12)**	**LHF (N=14)**	**HHF (N=13)**	**p-value**
Hepatic fat (%)	(3.96)	(7.26)	(15.9)	
Age (yrs)	14.6 (0.54)	13.9 (0.62)	13.7 (0.78)	0.615
Male (n, %)	**5 (41.7)**	**2 (14.3)***	**9 (69.2)*†**	**0.015**
BMI z-score	**1.95 (0.08)**	**2.01 (0.08)**	**2.30 (0.12)***	**0.030**
TG (mg/dl)	**72.8 (7.31)**	**122 (14.4)***	**201 (32.7)*†**	**0.001**
FFA (mEq/L)	**0.81 (0.08)**	**0.85 (0.07)**	**1.31 (0.80)***	**0.032**
Cholesterol (mg/dl)	157 (22.1)	161 (8.78)	176 (12.0)	0.354
LDL (mg/dl)	99.3 (8.28)	105 (6.56)	109 (9.69)	0.720
apoB (mg/dl)	61.2 (5.39)	65.5 (4.58)	72.8 (6.80)	0.369
Visceral adipose tissue (mm^2) ł	**8974 (1137)**	**7283 (708)***	**12924 (1379)*†**	**0.010**
HOMA-IR (mmol/L·pmol/L)	**3.92 (0.44)**	**6.89 (1.66)***	**11.3 (4.01)***	**0.028**
Adipose-IR (mmol/L·pmol/L)	**13.1 (1.60)**	**23.8 (5.99)**	**85.3 (46.7)*†**	**0.001**
Insulin (µU/L)	**17.1 (2.15)**	**27.7 (22.1)**	**46.3 (12.9)***	**0.012**
Adiponectin (µg/mL)	11.8 (1.26)	18.5 (2.1)	14.6 (2.08)	0.094
TNF-α (pg/mL)	4.5 (0.61)	4.9 (0.46)	6.3 (0.62)	0.187
ALT (U/L)	**17.3 (2.15)**	**17.1 (1.05)**	**94.3 (36.2)**	**<0.001**
AST (U/L)	**21.8 (1.11)**	**25.3 (1.43)**	**126 (77.0)**	**<0.001**
CRP (mg/L)	3.46 (1.03)	3.12 (0.64)	6.52 (2.18)	0.315

P-value generated by ANOVA or alternatively Kruskal-Wallis if not normally distributed;
* $p \leq 0.05$ as compared to NHF, † $p \leq 0.05$ as compared to LHF; ł NHF, n=10; LHF, N=13; HHF, N=8.
Significant p-values marked in bold
TG, triglycerides; FFA, free fatty acids; LDL, low-density lipoprotein; ApoB, apolipoprotein b; HOMA-IR, homeostatic model assessment for insulin resistance index; Adipo-IR, adipose insulin resistance index; TNF-α, tumor necrosis factor-α; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein

Table 2. Demographic and Metabolic Characteristics of Cohort 2

Variable	Fructose Group (n=9)	Glucose Group (n=12)
Age, yrs (Range)	11-18	11-18
Male/Female	3/6	7/5
BMI Z-Score	2.33 ± 0.55	2.10 ± 0.28
Hepatic Fat (%)	14.5 ± 5.4	13.4 ± 5.7
Triglycerides (mg/dl)	157.1 ± 104.6	177.1 ± 74.2
Cholesterol (mg/dl)	168.1 ± 27.6	180.3 ± 53.7
LDL (mg/dl)	108.4 ± 31.2	118.3 ± 43.8
ApoB (mg/dl)	63.4 ± 15.2	77.1 ± 26.1
HOMA-IR (mmol/L·pmol/L)	7.4 ± 2.9	7.3 ± 6.9
Adipo-IR (mmol/L·pmol/L)	28.6 ± 11.3	31.0 ± 22.8
Insulin (µU/L)	30.4 ± 12.8	30.9 ± 23.9

LDL, low-density lipoprotein; ApoB, apolipoprotein b; HOMA-IR, homeostatic model assessment for insulin resistance index; Adipo-IR, adipose insulin resistance index

Table 3. Parameter estimates from linear regression model with PAI-1 as dependent variable with nothing controlled

	Beta	(95% CI)	p-value
Hepatic Fat	**0.06**	**(0.04 – 0.09)**	**<0.0001**
Triglycerides	**0.003**	**(0.001 – 0.005)**	**0.001**
Free Fatty Acids	**0.47**	**(0.13 – 0.80)**	**0.007**
Cholesterol	0.004	(-0.001 – 0.01)	0.14
LDL	0.004	(-0.003 – 0.01)	0.24
ApoB	0.009	(-0.001 – 0.02)	0.078
VAT	0.00004	(-0.00002 – 0.00009)	0.20
HOMA-IR	**0.023**	**(0.0002 – 0.046)**	**0.048**
Adipo-IR	0.002	(-0.0002 – 0.004)	0.067
Insulin	**0.008**	**(0.001 – 0.01)**	**0.02**

LDL, low-density lipoprotein; ApoB, apolipoprotein b; VAT, visceral adipose tissue; HOMA-IR, homeostatic model assessment for insulin resistance index; Adipo-IR, adipose insulin resistance index

Table 4. Parameter estimates from linear regression model with PAI-1 as dependent variable and controlling for BMI

	Beta	(95% CI)	p-value
Hepatic Fat	**0.056**	**(0.027–0.085)**	**0.0004**
Triglycerides	**0.003**	**(0.001–0.005)**	**0.0009**
Free Fatty Acids	**0.40**	**(0.07–0.72)**	**0.02**
Cholesterol	0.004	(-0.001–0.009)	0.12
LDL	0.005	(-0.001–0.01)	0.13
ApoB	**0.009**	**(0.0005–0.02)**	**0.04**
VAT	0.00001	(-0.00005–0.00007)	0.63
HOMA-IR	0.015	(-0.01–0.04)	0.23
Adipo-IR	0.001	(-0.0009–0.004)	0.22
Insulin	0.005	(-0.002–0.01)	0.15

LDL, low-density lipoprotein; ApoB, apolipoprotein b; VAT, visceral adipose tissue; HOMA-IR, homeostatic model assessment for insulin resistance index; Adipo-IR, adipose insulin resistance index

Table 5. Parameter estimates from linear regression model with PAI-1 as dependent variable and controlling for HOMA-IR

	Beta	**(95% CI)**	**p-value**
Hepatic Fat	**0.057**	**(0.03 – 0.09)**	**0.0004**
Triglycerides	**0.003**	**(0.001 – 0.006)**	**0.005**
Free Fatty Acids	**0.42**	**(0.005 – 0.84)**	**0.048**
Cholesterol	0.002	(-0.004 – 0.009)	0.43
LDL	0.003	(-0.004 – 0.009)	0.46
ApoB	0.006	(-0.004 – 0.02)	0.23
VAT	0.00002	(-0.00004 – 0.00008)	0.48
Insulin	**0.04**	**(0.001 – 0.08)**	**0.04**

LDL, low-density lipoprotein; ApoB, apolipoprotein b; VAT, visceral adipose tissue; HOMA-IR, homeostatic model assessment for insulin resistance index

Table 6. Parameter estimates from linear regression model with PAI-1 as dependent variable and controlling for VAT

	Beta	(95% CI)	p-value
Hepatic Fat	**0.08**	**(0.03 – 0.12)**	**0.001**
Triglycerides	**0.005**	**(0.002 – 0.008)**	**0.001**
Free Fatty Acids	**0.63**	**(0.07 – 1.19)**	**0.03**
Cholesterol	0.005	(-0.002 – 0.01)	0.17
LDL	0.006	(-0.002 – 0.01)	0.13
ApoB	**0.01**	**(0.0003 – 0.02)**	**0.04**
HOMA-IR	0.03	(-0.02 – 0.07)	0.23
Adipo-IR	**0.01**	**(0.003 – 0.03)**	**0.02**
Insulin	0.009	(-0003 – 0.02)	0.13

LDL, low-density lipoprotein; ApoB, apolipoprotein b; VAT, visceral adipose tissue; HOMA-IR, homeostatic model assessment for insulin resistance index; Adipo-IR, adipose insulin resistance index

Table 7. Parameter estimates from linear regression model with PAI-1 as dependent variable and controlling for Hepatic Steatosis

	Beta	**(95% CI)**	**p-value**
Triglycerides	0.002	(-0.0006 – 0.004)	0.15
Free Fatty Acids	0.29	(-0.01 – 0.58)	0.06
Cholesterol	0.004	(-0.001 – 0.008)	0.12
LDL	0.005	(-0.0008 – 0.01)	0.09
ApoB	**0.008**	**(-0.0002 – 0.02)**	**0.05**
VAT	-0.00002	(-0.00008 – 0.00004)	0.42
HOMA-IR	0.01	(-0.008 – 0.03)	0.23
Adipo-IR	0.001	(-0.0007 – 0.003)	0.22
Insulin	0.004	(-0.002 – 0.01)	0.18

LDL, low-density lipoprotein; ApoB, apolipoprotein b; VAT, visceral adipose tissue; HOMA-IR, homeostatic model assessment for insulin resistance index; Adipo-IR, adipose insulin resistance index

Table 8. PAI-1 values (mean ± SE) by Beverage Group at Each Timepoint

	Fructose Group	**Glucose Group**
Baseline (0 weeks)	47.3 ± 7.8 ng/mL	51.3 ± 6.7 ng/mL
Two Weeks	48.7 ± 5.9 ng/mL	48.0 ± 9.7 ng/mL
Four Weeks	49.5 ± 7.0 ng/mL	50.9 ± 8.0 ng/mL

* Repeated Measures ANOVA (with interaction): F-Test = 1.59; p-value = 0.2

Table 9. Parameter Estimates from a Mixed Model ANOVA Regression with PAI-1 as Dependent Variable and Controlling for Individual*

Variable	Parameter Estimate	(95% CI)	P-Value
The difference between beverage groups at baseline	8.37 (ng/mL)	(-3.90–20.64)	0.08
The change in PAI-1 from baseline to 14 days among glucose consumers	-1.45 (ng/mL/14d)	(-5.50–2.60)	0.47
The change in PAI-1 from baseline to 28 days among glucose consumers	-2.19 (ng/mL/28d)	(-6.24–1.86)	0.28
The change in PAI-1 from baseline to 14 days among fructose consumers	3.35 (ng/mL/14d)	(-7.01–0.32)	0.07
The change in PAI-1 from baseline to 28 days among fructose consumers	-0.41 (ng/mL/28d)	(-4.07–3.26)	0.82
The change from baseline to 14 days comparing glucose to fructose consumers	4.80 (ng/mL/14d)	(-0.67–10.26)	0.08
The change from baseline to 28 days comparing glucose to fructose consumers	2.60 (ng/mL/28d)	(-2.86–8.06)	0.34

* Model: $E(\text{PAI-1}) = \beta_0 + \beta_1(\text{Beverage}) + \beta_2(\text{Timepoint1}) + \beta_3(\text{Timepoint2}) + \beta_4(\text{Beverage*Timepoint1}) + \beta_5(\text{Beverage*Timepoint2}) + \sum \gamma_i ID_j$, where Timepoint1 is 14 days and Timepoint2 is 28 days

Figure 1. Known Mechanism of the Action of PAI-1

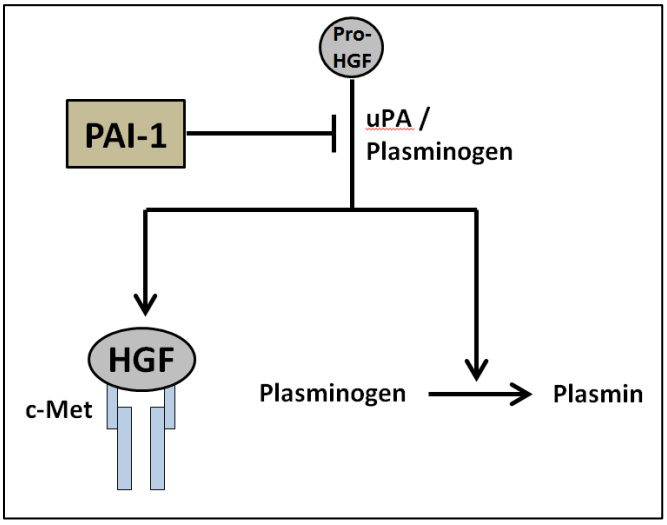


Figure 2. Visualization of Aim 1 and Aim 2 of the present study

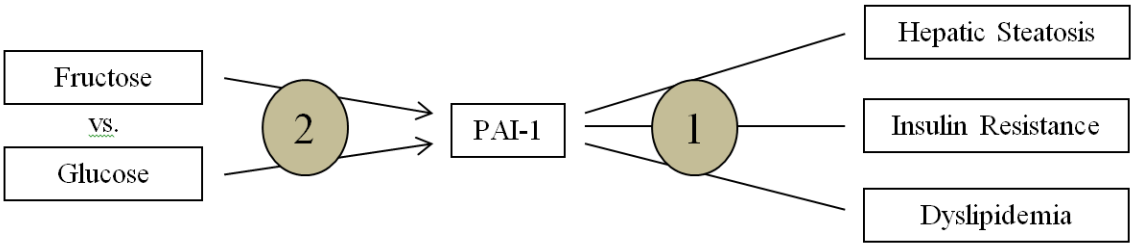


Figure 3. Flow diagram of the present study

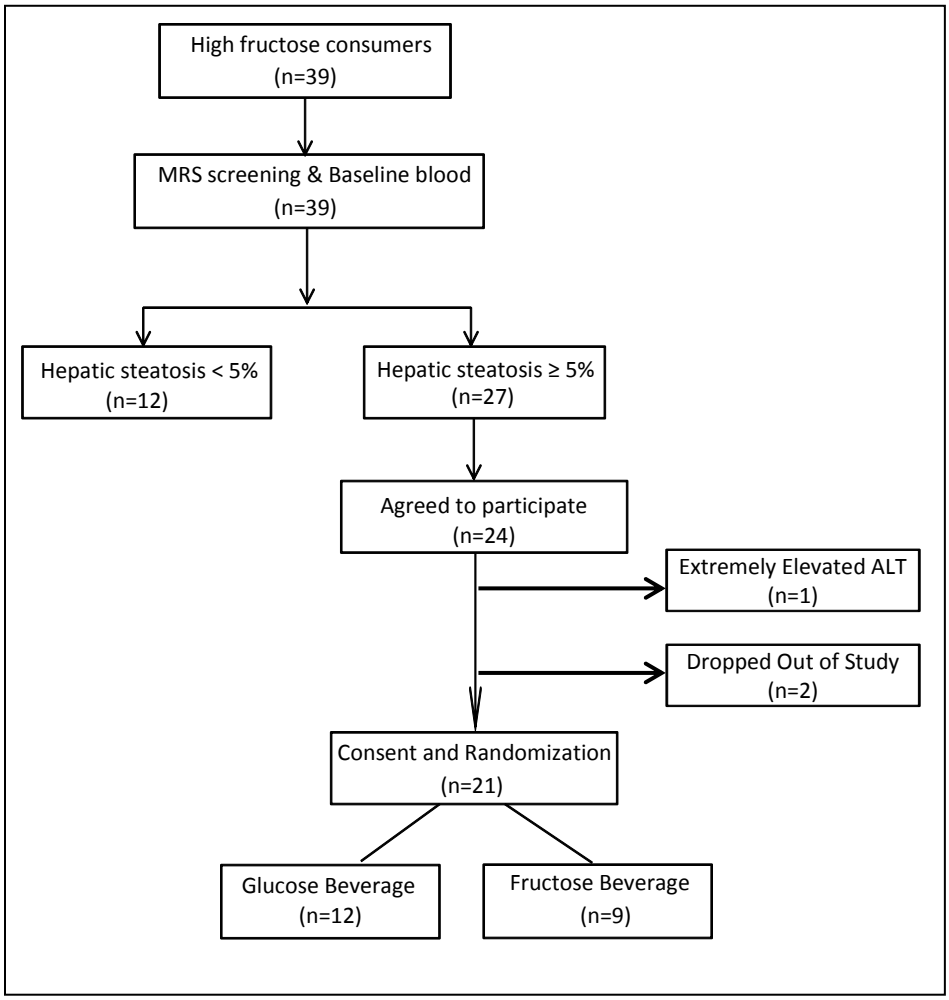


Figure 4. Diagrammatic Relationship between PAI-1 and Variables of Interest

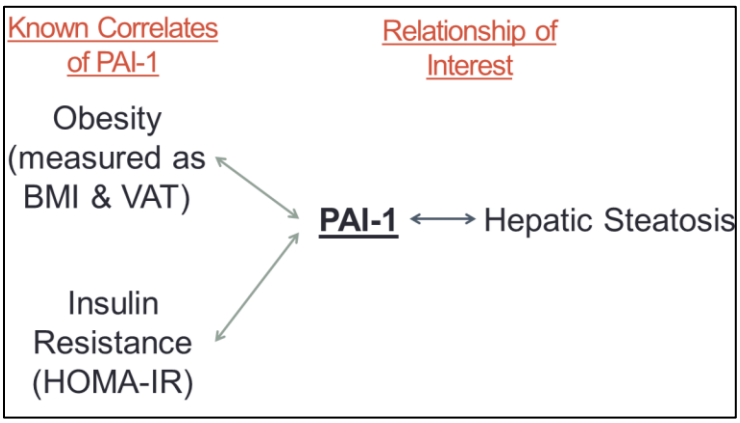
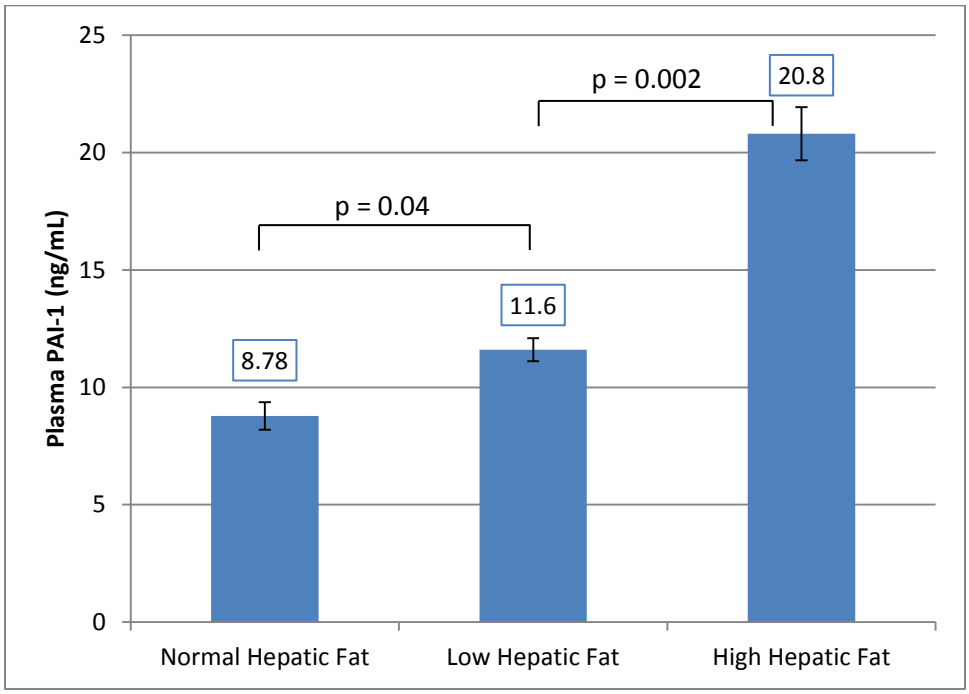


Figure 5. The trend of plasma PAI-1 concentration in Hispanic, obese children with normal, low, and high hepatic fat*



* p-value < 0.001 by Kruskal-Wallis' test comparing the 3 groups

Figure 6. Mean PAI-1 Levels over 4 Week Period between Beverage Groups

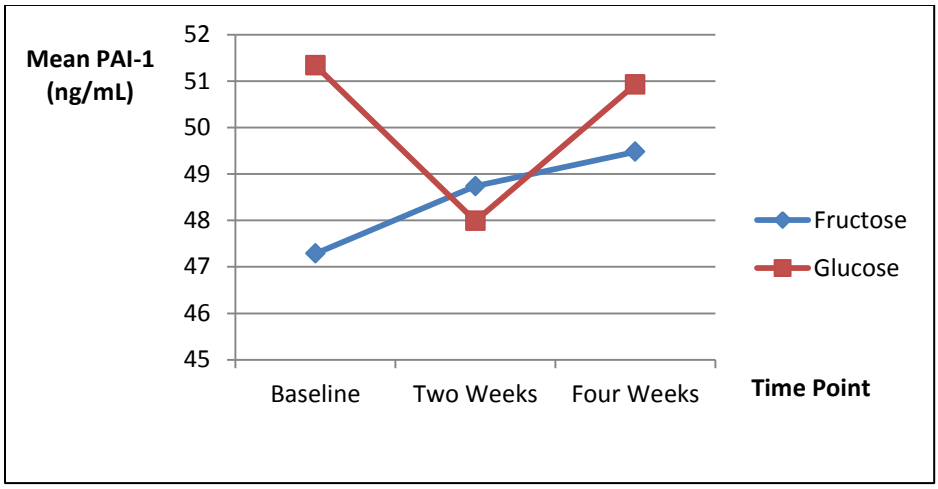


Figure 7. Trend of PAI-1 over the 4 Week Period by Individual within Fructose Group

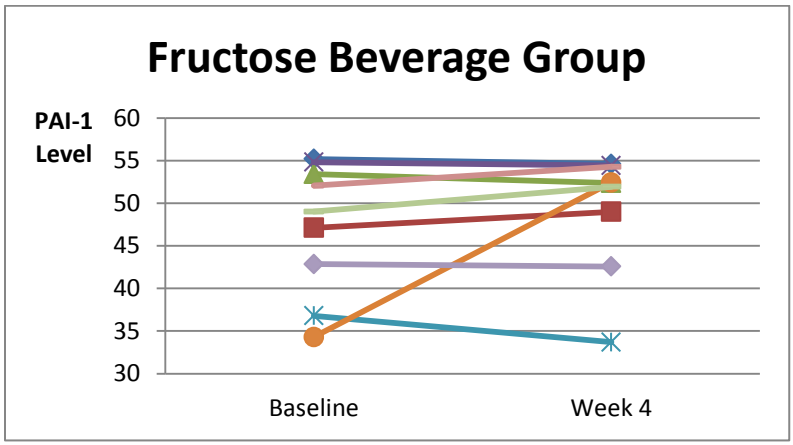
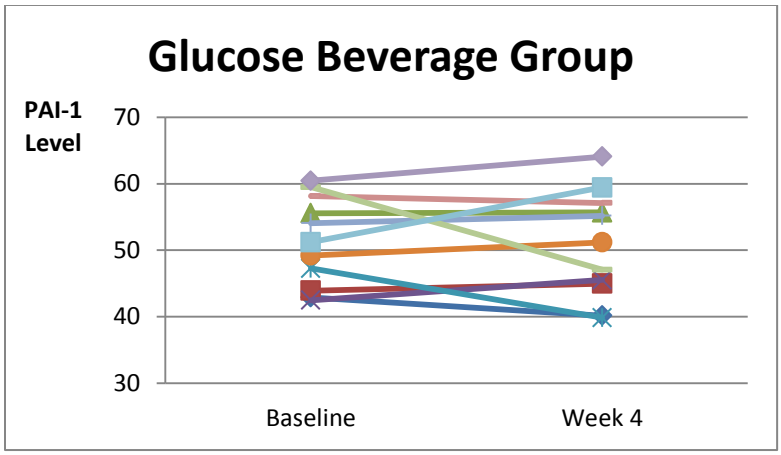


Figure 8. Trend of PAI-1 over the 4 Week Period by Individual within Glucose Group



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APPENDIX:

Tests for Normality of the Unedited and Log-Transformed Predictor and Outcome Variables

	Kolmogorov-Smirnov Test*	**Cramer-von Mises Test***	**Anderson-Darling Test***	**Skewness^**	**Kurtosis^**
PAI-1	0.079	0.033	0.032	1.22	2.26
Hepatic Fat	0.016	<0.005	<0.005	1.22	1.26
HOMA-IR	< 0.01	<0.005	<0.005	3.62	14.68
Adipo-IR	< 0.01	<0.005	<0.005	5.63	33.08
Log (PAI-1)	> 0.150	0.211	0.141	-0.80	1.40
Log (Hepatic Fat)	> 0.150	0.226	0.241	0.22	-0.96
Log (HOMA-IR)	0.029	0.022	0.017	0.88	1.92
Log (Adipo-IR)	0.078	0.026	0.028	1.38	3.99

* Note that in these tests for normality, the p-value is based on the assumption that the distribution is normal. So a low p-value indicates that you reject normality and your sample is non-normal.

^ Note that normal population is assumed if skewness or kurtosis is < 1. If it is > 1, then non-normality assumed.