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Nonalcoholic Fatty Liver Disease in Hispanic Youth With Dysglycemia: Risk for Subclinical Atherosclerosis?

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Context: Obese Hispanic adolescents (OHAs) with dysglycemia have increased cardiovascular disease risk burden.

Objective: To investigate if nonalcoholic fatty liver disease (NAFLD) confers added risk for endothelial dysfunction in these youth.

Design: Cross-sectional study.

Setting: Academic institution.

Participants: Thirty-six OHAs (15.3 ± 0.4 years), 20 with prediabetes and 16 with type 2 diabetes, with and without NAFLD.

Intervention: Evaluation of reactive hyperemia index (RHI) and augmentation index (AIx) by peripheral arterial tonometry; muscle, hepatic, and adipose tissue insulin sensitivity (IS; hyperinsulinemic-euglycemic clamp 80 mu/m^2/min, with [6,6 $^2H_2$]glucose and [$^2H_5$] glycerol); body composition; and abdominal and hepatic fat by magnetic resonance imaging/spectroscopy.

Outcome Measures: RHI and AIx.

Hypothesis: OHAs with dysglycemia and NAFLD have worse RHI and AIx vs those without NAFLD.

Results: The NAFLD (n = 23) and non-NAFLD (n = 13) groups were of similar age, sex, glycemic status, body mass index, % body fat and abdominal fat. The NAFLD group had higher hepatic fat ($P < 0.001$) lower skeletal muscle IS ($P = 0.01$), hepatic IS ($P = 0.01$), and adipose tissue IS ($P = 0.04$). The NAFLD vs non-NAFLD group had lower RHI (1.4 ± 0.05 vs 1.7 ± 0.09, $P = 0.002$), greater AIx (–6.0 ± 1.6 vs –12.0 ± 2.1, $P = 0.03$). Hepatic fat was inversely related to RHI ($r = –0.49$, $P = 0.002$) and positively related to AIx ($r = 0.45$, $P = 0.006$). Hepatic IS ($r = –0.42$, $P = 0.01$) and adipose IS ($r = –.54$, $P = 0.001$) correlated with arterial stiffness (AIx).

Abbreviations: AIx, augmentation index; AIx-75, augmentation index adjusted to a standard heart rate of 75 beats per minute; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; c-IMT, carotid intima-media thickness; CVD, cardiovascular disease; HDL, high-density lipoprotein; HEC, hyperinsulinemic-euglycemic clamp; HFF, hepatic fat fraction; hs-CRP, high-sensitivity C-reactive protein; ICAM-1, intercellular cell adhesion molecule-1; IGT, impaired glucose tolerance; IS, insulin sensitivity; LDL, low-density lipoprotein; MRS, magnetic resonance spectroscopy; NAFLD, nonalcoholic fatty liver disease; OGTT, oral glucose tolerance test; OHA, obese Hispanic adolescent; PAT, peripheral arterial tonometry; R_d, rate of disposal; RHI, reactive hyperemia index; T2DM, type 2 diabetes mellitus; VCAM-1, vascular cell adhesion molecule-1.

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Conclusion: In OHAs with dysglycemia, NAFLD is associated with worse endothelial function. RHI and AIx were related to hepatic fat content. Vascular stiffness was related to hepatic and adipose tissue insulin resistance.

Freeform/Key Words: NAFLD, hepatic fat, endothelial function, inflammatory markers, insulin resistance

Nonalcoholic fatty liver disease (NAFLD), comprising a spectrum of conditions ranging from steatosis to steatohepatitis and cirrhosis, represents the most common cause of chronic liver disease. The prevalence has increased significantly with the current obesity epidemic and is estimated to be between 20% and 30% in the general adult population [1] and ~10% in children [2, 3]. The prevalence varies among ethnic groups. In the Dallas Heart Study, fatty liver disease was most common in Hispanics and lowest in African Americans [4]. Also, the prevalence is higher in individuals with type 2 diabetes mellitus (T2DM; 70% to 80%) compared with the general population (~30%) [1, 5]. Hispanic children are disproportionately affected by the obesity epidemic with 38.1% of Hispanic children and adolescents 12 to 19 years of age found to be overweight/obese according to the 2011–2012 National Health and Nutrition Examination data [6]. They are also known to be at high risk for T2DM [7] and NAFLD [2, 8].

In population studies, NAFLD was found to be independently associated with increased risk of cardiovascular disease (CVD) [9]. In adults with T2DM, elevated liver enzymes were associated with abnormal brachial flow-mediated dilation, independent of whole body insulin sensitivity (IS) and other prognostic factors, suggestive of an increased CVD risk in adults with T2DM and liver disease [10]. In diet controlled adults with T2DM, those with NAFLD (by ultrasound) had greater carotid intima-media thickness (c-IMT) explained by insulin resistance index rather than liver fat [11]. Other studies questioned the added risk related to NAFLD in the setting of diabetes. c-IMT was not found to be higher in individuals with T2DM with vs without NAFLD, but hepatic steatosis was not associated with c-IMT [12]. Another study concluded that hepatic fat may be protective against atherosclerosis in 60-year-old adults with T2DM [13]. In children, increased hepatic fat has been associated with insulin resistance [14–16], prediabetes phenotype [17], higher inflammatory markers [17], and lower adiponectin [18]. Children with biopsy-proven NAFLD have significantly higher fasting insulin, triglycerides, cholesterol, blood pressure, and lower high-density lipoprotein (HDL) compared with obese peers with no NAFLD [19].

The role of NAFLD as an added risk for subclinical atherosclerosis in obese youth with altered glucose metabolism, beyond the effect of hyperglycemia is not clear. This study aimed to investigate whether the presence of NAFLD has an additive effect on subclinical endothelial dysfunction, an early biomarker of atherosclerosis, in obese Hispanic adolescents (OHAs) with prediabetes and T2DM. We hypothesized that (1) Hispanic youth with dysglycemia and NAFLD have worse endothelial function compared with those without NAFLD and (2) this endothelial dysfunction is related to insulin resistance and inflammation.

1. Research Design and Methods

A. Study Subjects

Thirty-six overweight/OHAs [body mass index (BMI) >85th percentile, 11 to 19 years of age] with impaired glucose regulation were studied. They were recruited from Texas

Children's Diabetes and Hepatology Centers and through advertisement in the community. Sixteen subjects had a diagnosis of T2DM and 20 subjects had prediabetes, including impaired fasting glucose (n = 8), impaired glucose tolerance (IGT; n = 6), or combined impaired fasting glucose and IGT (n = 6) based on 2-hour oral glucose tolerance test (OGTT) results, according to the American Diabetes Association criteria [20]. The criterion for enrollment into the NAFLD category was based on elevated liver transaminases with alanine aminotransferase (ALT) value greater than 40 U/L (approximately 1.5 times the normal value for children) [21] for up to 6 months prior to the study, after exclusion of other causes of liver disease by the treating hepatologist [22]. NAFLD status was confirmed by an elevated hepatic fat fraction (HFF) greater than 5.5% on magnetic resonance spectroscopy (MRS) [17, 18], performed after enrollment in the study. Non-NAFLD was defined as having liver transaminases less than 40 U/L and HFF less than 5.5% on MRS. Eighteen children in the NAFLD group had elevated transaminases and elevated liver fat. Also classified with NAFLD were three children recruited as controls who had elevated HFF >5.5% despite ALT <40 U/L, and two additional subjects with significant elevation (>2 standard deviations of upper limit of normal) of ALT levels alone due to unavailable MRS data (data lost secondary to technical error). Of the 36 adolescents, 23 were thus classified as having NAFLD. Puberty was assessed by a pediatric endocrinologist using Tanner staging criteria. All participants were pubertal (Tanner stages III to V) and nonsmokers. Individuals on chronic medications that may affect metabolic function (*e.g.*, steroids, antipsychotics) were excluded. Youth with T2DM had to be in adequate glycemic control (HbA1c less than 8%). The mean duration of T2D was 22.21 ± 5.5 months (mean ± standard error of the mean). Eight youth with T2DM were treated with metformin, five were maintained on insulin ± metformin, and three were on lifestyle changes, with no difference in diabetes duration or therapy in those with NAFLD vs no NAFLD. Metformin and long acting insulin were discontinued for 24 hours prior to the OGTT/EndoPAT testing and 48 hours prior to the clamp studies, as before [23]. Short acting insulin was used as needed to maintain glycemic control, up to 6 hours prior to the clamp or OGTT. All studies were approved by the Institutional Review Board of Baylor College of Medicine. Informed consent and children's assent were obtained. Clinical characteristics of the study subjects are summarized in Table 1.

2. Methods

A. Fasting Blood Measurements

ALT and aspartate aminotransferase (AST), HbA1c, fasting lipid profile [cholesterol, triglycerides, HDL, low-density lipoprotein (LDL)], adiponectin and markers of inflammation including high-sensitivity C-reactive protein (hs-CRP) and biomarkers of endothelial dysfunction including vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) were determined to assess the atherogenic profile.

B. Oral Glucose Tolerance Test

Participants ingested a solution containing 1.75 g/kg body weight, maximum of 75.0 g of dextrose. Blood samples were obtained at 15 minutes before; 0 minutes before; and 15, 30, 60, 90, and 120 minutes after the ingestion to determine plasma glucose and insulin.

C. Body Composition

Body composition was assessed by dual-energy X-ray absorptiometry scan [24].

Table 1. Anthropometric and Metabolic Characteristics in OHA With Dysglycemia and NAFLD vs Non-NAFLD

	Non-NAFLD (n = 13)	NAFLD (n = 23)	*P* Value
Sex	3 F/10 M	13 F/10 M	0.08
Prediabetes/T2DM	8/5	12/11	0.6
Age, y	15.7 ± 0.4	15.2 ± 0.5	0.5
BMI, kg/m^2	34.0 ± 1.3	36.2 ± 1.1	0.2
BMI *z* score	2.2 ± 0.1	2.3 ± 0.06	0.6
Body fat, %	36.4 ± 2.2	41.1 ± 1.1	0.07
Waist circumference, cm	105.0 ± 3.4	110.4 ± 3.4	0.3
Total abdominal fat, cm^2	573.7 ± 55.6	634.6 ± 34.4	0.3
Subcutaneous abdominal fat, cm^2	478.0 ± 52.0	527.7 ± 31.8	0.4
Visceral abdominal fat, cm^2	95.7 ± 8.4	106.9 ± 5.9	0.3
Hepatic fat fraction, %	3.2 ± 0.4	10.4 ± 1.1	<0.01
HbA1c, %	5.6 ± 0.1	5.9 ± 0.1	0.06
HbA1c, mmol/mol	38 ± 1.1	41 ± 1.1	
ALT, U/L	26.2 ± 3.5	80.6 ± 10.7	0.001
Males	26.2 ± 4.3	94.5 ± 20.3	0.008
Females	26.6 ± 7.5	69.9 ± 10.7	0.07
AST, U/L	23.2 ± 2.5	52.4 ± 7.6	0.001
Males	20.2 ± 1.5	56.2 ± 13.3	0.024
Females	33.3 ± 7.5	49.5 ± 9.2	0.4
Total cholesterol, mg/dL	138.9 ± 5.1	165.0 ± 5.3	0.003
LDL cholesterol, mg/dL	76.6 ± 5.2	98.0 ± 4.6	0.005
Non-HDL cholesterol, mg/dL	98.8 ± 4.5	124.8 ± 5.1	0.001
Triglycerides, mg/dL	111.1 ± 13.4	137.3 ± 11.6	0.1
Systolic blood pressure, mm Hg	122.8 ± 2.8	118.5 ± 2.0	0.2
Diastolic blood pressure, mm Hg	73.1 ± 2.6	74.3 ± 1.3	0.2
Adiponectin, mg/L	18.4 ± 5.1	11.0 ± 1.9	0.1
hs-CRP, mg/L	2.2 ± 0.6	5.6 ± 1.2	0.02
s-ICAM-1, ng/mL	121.5 ± 9.5	166.4 ± 12.8	0.02
s-VCAM-1, ng/mL	452.1 ± 44.0	663.4 ± 37.8	0.001
E-selectin, ng/mL	54.9 ± 4.3	79.3 ± 8.1	0.01

Visceral and subcutaneous abdominal fat data were available in 12 of the non-NAFLD group and 19 of the NAFLD group secondary to technical problem in data acquisition and storage.

D. Abdominal Fat Partition and Hepatic Fat Determination

Intra-abdominal total fat content and the partition into subcutaneous and visceral adipose tissue were assessed using by magnetic resonance imaging scan using a 1.5 Tesla magnet (Philips, Achieva R3.2.1) at Texas Children's Hospital radiology department. Hepatic fat content was measured by proton MRS. We used the single voxel point-resolved spectroscopy [25] technique with a 16-channel SENSE-XL-Torso coil. A voxel ($25 \times 25 \times 25$ mm^3) was placed avoiding blood vessels and intrahepatic bile ducts with the following parameters (repetition time = 2500 ms, echo time = 31 ms). To achieve adequate signal-to-noise ratio, 64 acquisitions with a measuring time of 160 seconds were acquired without water suppression and averaged for intrahepatic lipid (%) calculation. Spectra were analyzed using a linear combination model [26]. Absolute concentrations of intrahepatic lipid were obtained from areas under curves of lipid at 0.9, 1.3 and 1.6 ppm, using tissue water content as an internal reference. The intrahepatic lipid is calculated using the following equation: Intrahepatic lipid (%) = 100 × lipid / (water + lipid).

E. Endothelial Function

Changes in pulse wave velocity during reactive hyperemia were measured using peripheral arterial tonometry (PAT; Itamar Medical Ltd). This is a noninvasive technology that captures plethysmographic recording of the finger arterial pulse wave velocity with pneumatic probes

inserted on the index fingers of both hands [27]. A reactive hyperemia index (RHI) is calculated as the ratio of the average amplitude of the PAT signal starting 1 minute after cuff deflation divided by the average amplitude of the PAT signal of 3.5-minute period before cuff inflation, normalized to the signal from the contralateral control finger [24]. The augmentation index (AIx) is a measure of arterial stiffness. It is usually a negative number calculated as the difference between the early (P1) and late (P2) systolic peaks of the pulse wave relative to the early peak wave (P2 – P1 / P1) expressed as a percentage [28], and adjusted to a standard heart rate of 75 beats per minute (AIx-75) [29]. A higher AIx (less negative number) reflects greater arterial stiffness.

E-1. Blood pressure

Blood pressure was measured using an automated sphygmomanometer in the morning of the study.

F. Clamp Studies and In Vivo Insulin Sensitivity

F-1. Basal substrate turnover

Basal hepatic glucose production and total body lipolysis was evaluated by the use of stable isotopes [6,6-$^2H_2$] glucose and [$^2H_5$] glycerol started 120 minutes before starting the clamp experiment [30]. Arterialized blood samples for glucose, insulin, and isotopic enrichment are obtained before the start of the isotope infusion and every 15 minutes from 60 to 120 minutes. Turnover calculations are made over the last 30 minutes of the isotopic steady state to determine hepatic and adipose tissue IS (see Calculations section).

F-2. In vivo insulin sensitivity

Following the baseline isotopic infusion period, a hyperinsulinemic-euglycemic clamp (HEC) was performed [30] to evaluate *in vivo* insulin action. Intravenous insulin (Humulin; Lilly Indianapolis, IN) was infused at 80 mU/m^2/min and a variable rate of infusion of 20% dextrose enriched with [6,6-$^2H_2$] glucose based on plasma glucose determinations every 5 minutes was used to maintain plasma glucose clamped at ~100 mg/dL. 15 of the NAFLD and 5 of the non-NAFLD group had a 3.5-hour two-step HEC (low dose insulin 16 mU/m^2/min for 1.5 hours followed by high dose 80 mU/m^2/min for 2 hours). For this current analysis, data from the steady state of the high-dose insulin clamp are used for determination of peripheral (muscle) IS. This is possible because steady-state glucose (102.4 ± 0.5 vs 102.8 ± 0.9 mg/dL), insulin (265.2 ± 9.5 vs 252.7 ± 12.4 μU/mL) and enrichment of [6,6-$^2H_2$] glucose (3.1 ± 0.12% vs 2.9 ± 0.13%) were similar at the end of the 80 mU/m^2/min 3.5-hour two-step clamp to that of the 80 mU/m^2/min 3-hour HEC. The rate of glucose infusion is determined based on arterialized plasma glucose measurements every 5 minutes. Blood is sampled every 10 to 15 minutes for determination of insulin levels [30].

F-3. Calculations

Substrate turnover at baseline is calculated during the last 30 minutes of the fasting 2-hour isotopic infusion period according to steady state tracer dilution equations to determine hepatic glucose production and basal rate of lipolysis. Insulin-stimulated glucose rate of disposal (R_d) is calculated during the last 30 minutes of the hyperinsulinemic-euglycemic clamp, as equivalent to the rate of exogenous glucose infusion and expressed per fat free mass (mg/min/kg$_{FFM}$). Peripheral skeletal muscle IS was calculated by dividing the R_d by the

steady-state clamp insulin concentration and expressed per FFM (mg/min/kg_{FFM} per μU/mL). Hepatic IS was calculated as the inverse of the product of hepatic glucose production and the fasting plasma insulin concentration [31]. Adipose tissue IS is calculated as the inverse of product of glycerol rate of appearance in plasma and fasting plasma insulin concentration [32].

G. Biochemical Measurements

Plasma glucose was measured with a glucose analyzer (Yellow Springs Instrument Co., Yellow Springs, OH), Insulin levels were measured by electrochemiluminescence immunoassays (Elecsys 2010, Roche Diagnostics, Indianapolis, IN). HbA1c was measured using Tinaquant HbA1c immunoassay from Roche and lipids were measured using the standards of the Centers for Disease Control and Prevention at Labcorps, Inc. Adiponectin and adhesion molecules ICAM-1, VCAM-1, and E-selectin concentrations were quantified using Magpix (Milliplex MAP) immunoassay (EMD Millipore Corporation, Billerica, MA). hs-CRP levels were measured at Esoterix Inc., Calabasas Hills, CA.

H. Statistics

The normality of the distribution of the variables was examined. Statistical analyses were performed using the t test or the Mann–Whitney U nonparametric equivalent for two group comparisons. The nonparametric variables were RHI, BMI z score, HbA1c, ALT, AST, hepatic fat fraction, rate of glucose disposal, and IS. Pearson or Spearman's correlation analyses were used to evaluate bivariate relationships. χ^2 test was used for the comparison of categorical variables. Data are presented as mean ± standard error of the mean. Two-tailed $P \leq 0.05$ was considered statistically significant. Power estimates based on the literature [27] indicated that a sample size of 13 subjects per group was needed to detect a 30% difference in mean RHI (standard deviation = 0.25) between groups with vs without NAFLD, with $P = 0.8$, $\alpha = 0.05$.

3. Results

A. Subjects Characteristics

The study population of adolescents with dysglycemia was divided into two groups according to the presence or absence of NAFLD. The two groups had a similar proportion of children with prediabetes and T2DM. The two groups were similar with respect to age, sex, and pubertal stage. They had similar BMI, BMI percentile for age and sex, BMI z score, and percent body fat. They also had similar abdominal adiposity including waist circumference, total, subcutaneous, and visceral abdominal fat. By design, the group with NAFLD had significantly higher hepatic fat content and higher transaminases (ALT and AST; Table 1).

B. Endothelial Function and CVD Risk Biomarkers in NAFLD vs Non-NAFLD Subjects

Triglycerides and systolic and diastolic blood pressures were not significantly different between the two groups. Total, LDL and non-HDL cholesterol, circulating inflammatory markers including hs-CRP, ICAM, VCAM, and e-selectin were significantly higher in the NAFLD group (Table 1). Youth with NAFLD compared with those without NAFLD had lower RHI ($P = 0.002$) and greater (worse) AIx ($P = 0.002$) and AIx-75 ($P = 0.03$; Fig. 1). The group differences in RHI (1.4 ± 0.06 vs 1.7 ± 0.08, $P = 0.002$) and AIx (–3.9 ± 1.4 vs –10.9 ± 1.9, $P = 0.006$) persisted after adjustment of sex. Also, when the prediabetes and T2DM groups were analyzed separately, the NAFLD group continued to have lower RHI (1.40 ± 0.07 vs 1.68 ± 0.12, $P = 0.04$) and greater AIx (–2.3 ± 2.1 vs –9.6 ± 1.3, $P = 0.009$)

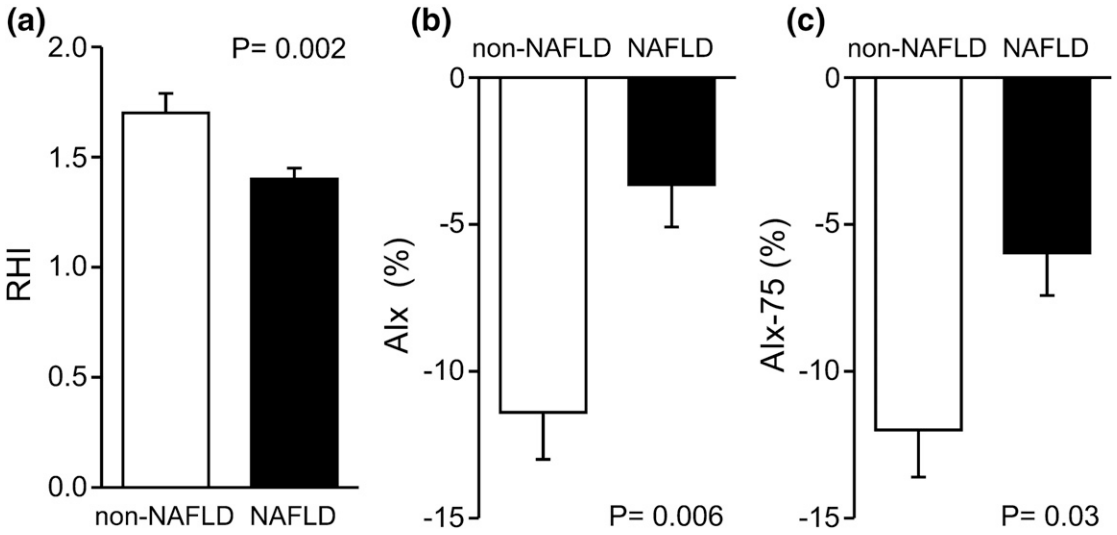

Figure 1. Endothelial function measures (RHI, AIx and AIx-75) in OHA with dysglycemia and NAFLD vs non-NAFLD.

than the non-NAFLD group in those with prediabetes, as well as lower RHI (1.42 ± 013 vs 1.82 ± 0.03, $P = 0.03$) and greater AIx (–5.2 ± 2.0 vs –14.2 ± 3.4, $P = 0.03$) in those with T2DM.

C. Fasting and HEC Metabolic Characteristics of NAFLD vs Non-NAFLD

Fasting glucose and hepatic glucose production were not significantly different between the NAFLD and non-NAFLD groups, whereas glycerol rate of appearance was higher in the NAFLD group (Table 2). Fasting insulin levels tended to be higher and both hepatic IS and adipose tissue IS were significantly lower in the NAFLD compared with the non-NAFLD group (Table 2). Peripheral skeletal muscle glucose R_d and IS were also significantly lower in the NAFLD vs non-NAFLD groups. The difference in IS persisted after adjusting for sex (data not shown).

Table 2. Metabolic Parameters at Baseline Fasting State and at the Steady State of the Hyperinsulinemic-Euglycemic Clamp in OHA With Dysglycemia and NAFLD vs Non-NAFLD

	Non-NAFLD	NAFLD	*P* Value
Fasting measures			
Fasting glucose, mg/dL	105.7 ± 1.9	111.5 ± 4.0	0.2
Fasting insulin, μU/mL	28.0 ± 2.8	35.5 ± 2.7	0.08
Hepatic glucose production, mg/kg.min	2.1 ± 0.11	2.2 ± 0.07	0.7
Hepatic IS, mg/kg/min. μU/mL^{-1}	19.0 ± 1.9	13.4 ± 1.2	0.012
Gycerol rate of appearance, μmol/kg.min	1.9 ± 0.2	2.6 ± 0.2	0.03
Adipose tissue-IS, μmol/kg.min. μU/mL^{-1}	22.4 ± 4.0	13.3 ± 1.5	0.045
FFA, meq/L	0.67 ± 0.07	0.85 ± 0.06	0.06
Hyperinsulinemic-euglycemic clamp measures			
Steady state plasma glucose, mg/dL	101.9 ± 0.6	103.0 ± 0.7	0.4
Steady state plasma insulin, μU/mL	245.3 ± 9.8	267.1 ± 10.5	0.2
Insulin-stimulated glucose disposal, mg/kg.min	5.8 ± 0.6	4.1 ± 0.4	0.02
Insulin-stimulated glucose disposal, mg/kg$_{FFM}$.min	9.4 ± 0.8	7.2 ± 0.7	0.049
IS, mg/kg.min per μU/mL	2.4 ± 0.3	1.6 ± 0.2	0.014
IS per fat free mass, mg/kg$_{FFM}$.min per μU/mL	3.8 ± 0.4	2.8 ± 0.3	0.03

Glycerol rate of appearance and adipose tissue IS were missing in one subject in the non-NAFLD group (isotope infusion rate error).

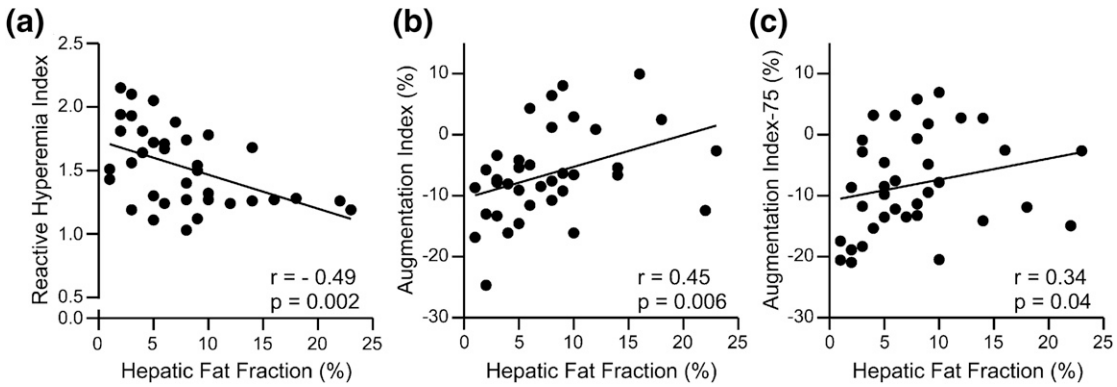

Figure 2. Relationship of hepatic fat fraction (%) to the (a) RHI, (b) AIx, and (c) AIx-75. For AIx, a higher (less negative) number reflects greater arterial stiffness.

D. Relationship of NAFLD and Insulin Sensitivity Measures to Endothelial and Circulating Biomarkers

Hepatic fat content was positively related to ALT (r = 0.49, P = 0.002) and AST (r = 0.40, P = 0.02) levels, and positively related to total cholesterol (r = 0.53, P = 0.001), LDL cholesterol (r = 0.34, P = 0.04) and non HDL-cholesterol (r = 0.47, P = 0.004), but not with triglycerides (P = 0.1).

Hepatic fat content was inversely related to RHI (r = –0.49, P = 0.002), and positively related to AIx (r = 0.45 P = 0.006), AIx-75 (r = 0.34, P = 0.04; Fig. 2), and to VCAM-1 (r = 0.45, P = 0.03). ALT and AST were similarly inversely related to RHI (r = –0.45, P = 0.006 and r = –0.46, P = 0.005, respectively) and positively related to AIx-75 (r = 0.44, P = 0.008 and r = 0.37, P = 0.025, respectively) and to hs-CRP (r = 0.40, P = 0.01 and r = 0.33, P = 0.04, respectively).

Among the inflammatory markers, VCAM-1 was inversely related to RHI (r = –0.37, P = 0.046), and positively related to AIx-75 (r = 0.42, P = 0.02). E-selectin was also inversely related to RHI (r = –0.35, P = 0.04).

Hepatic IS and adipose tissue IS were inversely related to AIx-75 (r = –0.42, P = 0.01 and r = –0.54, P = 0.001, respectively), but not to RHI. Peripheral IS did not directly relate to RHI or AIx.

4. Discussion

In OHAs with dysglycemia (prediabetes and T2DM), the presence of NAFLD is associated with worse endothelial function, a biomarker of subclinical atherosclerosis, as indicated by lower reactive hyperemia index (nitric oxide dependent vascular function), higher augmentation index (a measure of peripheral vascular stiffness) [27, 29], and higher levels of circulating inflammatory markers. These measures of vascular function were related to the hepatic fat content, independent of total body and visceral adiposity, and other CVD risk markers of glycemia, and blood pressure. These findings support the hypothesis that ectopic fat deposition in the liver may have a detrimental effect on the risk for subclinical atherosclerosis in this high-risk group of youth.

Our findings of worse endothelial function in youth with NAFLD are consistent with reports of increased c-IMT and greater prevalence of carotid plaques in obese adult patients with NAFLD diagnosed via liver biopsy, compared with controls of similar age, sex, and BMI but normal liver ultrasound and liver function tests [33]. In Hispanic adolescents, increased hepatic fat is associated with worse lipoprotein profile [34]. Obese 10- to 12-year-old Italian children with ultrasound diagnosed NAFLD and elevated ALT were found to have impaired flow mediated dilation of the brachial artery compared with obese and normal weight controls

independent of other CVD risk factors [35]. On the other hand, an Australian study did not find a relationship between NAFLD (by MRS) and arterial stiffness or c-IMT in severely obese children, primarily of European descent [36]. In 17-year-old adolescents, presence of NAFLD (ultrasound) was found to be associated with increased pulse wave velocity (by applanation tonometry) only in the presence of a cluster of cardiometabolic risk factors and AIx-75 was higher in males but not in females with NAFLD [37]. Our study extends these findings to demonstrate that NAFLD predisposes to greater risk of vascular dysfunction in the setting of dysglycemia in high risk youth with prediabetes and T2DM.

One possible mechanism linking NAFLD to subclinical atherosclerosis is the contribution of NAFLD to insulin resistance. Our youth with dysglycemia and NAFLD have significantly worse multiorgan insulin resistance including peripheral, hepatic and adipose tissue insulin resistance compared with youth without NAFLD, of similar total body and visceral fat. This supports the role of hepatic fat in impairment of glucose and lipid metabolism. This is consistent with findings in adults, where presence of NAFLD and either prediabetes or T2DM was associated with significant hepatic insulin resistance, compared with individuals of similar body composition without NAFLD [5]. Similarly, in another study of adults with T2DM, adipose tissue IS and hepatic tissue IS were lower in those with more severe liver disease [38]. In pediatric studies, obese adolescents with NAFLD had significantly lower peripheral IS with lower [15] or no difference in hepatic IS [39] compared with those without NAFLD. The difference between our findings and those of these two pediatric studies is likely due to different study populations with different ethnic background vs only Hispanic youth in the current study and, a combination of normal glucose tolerance and IGT in the former studies [34] vs uniformly impaired glucose regulation in our study population. Importantly, we found decreased IS at multiple organ level in youth with NAFLD. A direct relationship between skeletal muscle IS and RHI which we previously observed in a group of normal weight and overweight adolescents [29] was not readily apparent in the current study, likely because of less variance in skeletal muscle IS between the two groups of obese youth. However, insulin resistance at the level of the liver and adipose tissue was related to vascular stiffness, a relationship that is mediated by hepatic fat.

Other mediators of atherosclerosis in NAFLD include oxidative stress and the release of proatherogenic factors from the liver (C-reactive protein and other inflammatory cytokines) [1, 40]. Consistent with this, our youth with NAFLD had higher levels of hs-CRP, ICAM, VCAM-1, and e-selectin compared with the non-NAFLD group, and there was a direct relationship between VCAM-1 and e-selectin and endothelial function measures. The lack of a more uniform association between inflammatory markers and vascular measures is not clear. Similarly, others reported no association between hs-CRP and cardiac dysfunction despite significantly higher levels of hs-CRP in NAFLD vs controls [41]. Consistent with other studies, hepatic fat content was associated with serum ALT and AST levels as well as with non-HDL cholesterol levels [3, 42]. The transaminase levels, biomarkers of NASH, had a strong relationship to the functional measures of endothelial function (RHI and AIx-75).

All the subjects in this study were of Hispanic extraction with prediabetes or T2DM, and further studies are needed to demonstrate generalizability of the results. Nevertheless, our results clarify conflicting findings in the literature as to the contribution of NAFLD to CVD risk in individuals with dysglycemia [12, 13], using direct measures of vascular function and *in vivo* evaluation of IS at the level of the liver, adipose tissue, and skeletal muscle. There were more males than females in the non NAFLD group, even though the sex distribution difference between the two groups did not reach statistical significance. Adjusting for sex didn't change the difference in vascular measures or IS between the two groups. Larger studies are needed to further explore any possible sex related differences in vascular risk or IS.

In summary, NAFLD and the associated hepatic and adipose tissue insulin resistance are related to the impairment of vascular reactivity and increased vascular stiffness in OHAs with dysglycemia. In the high risk group of Hispanic youth with dysglycemia, the presence of

NAFLD is an important risk factor for subclinical atherosclerosis. This supports the importance of surveillance for liver disease in these high risk children, as an indicator of increased risk for early atherogenesis.

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References and Notes

1. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? *Diabetologia*. 2008;**51**(11):1947–1953.
2. Schwimmer JB, Deutsch R, Kahen T, Lavine JE, Stanley C, Behling C. Prevalence of fatty liver in children and adolescents. *Pediatrics*. 2006;**118**(4):1388–1393.
3. Alkhouri N, Carter-Kent C, Elias M, Feldstein AE. Atherogenic dyslipidemia and cardiovascular risk in children with nonalcoholic fatty liver disease. *Clin Lipidol*. 2011;**6**(3):305–314.
4. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology*. 2004;**40**(6):1387–1395.
5. Ortiz-Lopez C, Lomonaco R, Orsak B, Finch J, Chang Z, Kochunov VG, Hardies J, Cusi K. Prevalence of prediabetes and diabetes and metabolic profile of patients with nonalcoholic fatty liver disease (NAFLD). *Diabetes Care*. 2012;**35**(4):873–878.
6. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. *JAMA*. 2014;**311**(8):806–814.
7. Dabelea D, Bell RA, D'Agostino RB Jr, Imperatore G, Johansen JM, Linder B, Liu LL, Loots B, Marcovina S, Mayer-Davis EJ, Pettitt DJ, Waitzfelder B; Writing Group for the SEARCH for Diabetes in Youth Study Group. Incidence of diabetes in youth in the United States. *JAMA*. 2007;**297**(24):2716–2724.
8. Liska D, Dufour S, Zern TL, Taksali S, Calí AM, Dziura J, Shulman GI, Pierpont BM, Caprio S. Interethnic differences in muscle, liver and abdominal fat partitioning in obese adolescents. *PLoS One*. 2007;**2**(6):e569.
9. Stepanova M, Younossi ZM. Independent association between nonalcoholic fatty liver disease and cardiovascular disease in the US population. *Clin Gastroenterol Hepatol*. 2012;**10**(6):646–650.
10. Schindhelm RK, Diamant M, Bakker SJ, van Dijk RA, Scheffer PG, Teerlink T, Kostense PJ, Heine RJ. Liver alanine aminotransferase, insulin resistance and endothelial dysfunction in normotriglyceridaemic subjects with type 2 diabetes mellitus. *Eur J Clin Invest*. 2005;**35**(6):369–374.
11. Targher G, Bertolini L, Padovani R, Poli F, Scala L, Zenari L, Zoppini G, Falezza G. Non-alcoholic fatty liver disease is associated with carotid artery wall thickness in diet-controlled type 2 diabetic patients. *J Endocrinol Invest*. 2006;**29**(1):55–60.

12. Petit JM, Guiu B, Terriat B, Loffroy R, Robin I, Petit V, Bouillet B, Brindisi MC, Duvillard L, Hillon P, Cercueil JP, Verges B. Nonalcoholic fatty liver is not associated with carotid intima-media thickness in type 2 diabetic patients. *J Clin Endocrinol Metab*. 2009;**94**(10):4103–4106.
13. Loffroy R, Terriat B, Jooste V, Robin I, Brindisi MC, Hillon P, Vergès B, Cercueil JP, Petit JM. Liver fat content is negatively associated with atherosclerotic carotid plaque in type 2 diabetic patients. *Quant Imaging Med Surg*. 2015;**5**(6):792–798.
14. Deivanayagam S, Mohammed BS, Vitola BE, Naguib GH, Keshen TH, Kirk EP, Klein S. Nonalcoholic fatty liver disease is associated with hepatic and skeletal muscle insulin resistance in overweight adolescents. *Am J Clin Nutr*. 2008;**88**(2):257–262.
15. D'Adamo E, Cali AM, Weiss R, Santoro N, Pierpont B, Northrup V, Caprio S. Central role of fatty liver in the pathogenesis of insulin resistance in obese adolescents. *Diabetes Care*. 2010;**33**(8):1817–1822.
16. Perseghin G, Bonfanti R, Magni S, Lattuada G, De Cobelli F, Canu T, Esposito A, Scifo P, Ntali G, Costantino F, Bosio L, Ragogna F, Del Maschio A, Chiumello G, Luzi L. Insulin resistance and whole body energy homeostasis in obese adolescents with fatty liver disease. *Am J Physiol Endocrinol Metab*. 2006;**291**(4):E697–E703.
17. Cali AM, De Oliveira AM, Kim H, Chen S, Reyes-Mugica M, Escalera S, Dziura J, Taksali SE, Kursawe R, Shaw M, Savoye M, Pierpont B, Constable RT, Caprio S. Glucose dysregulation and hepatic steatosis in obese adolescents: is there a link? *Hepatology*. 2009;**49**(6):1896–1903.
18. Burgert TS, Taksali SE, Dziura J, Goodman TR, Yeckel CW, Papademetris X, Constable RT, Weiss R, Tamborlane WV, Savoye M, Seyal AA, Caprio S. Alanine aminotransferase levels and fatty liver in childhood obesity: associations with insulin resistance, adiponectin, and visceral fat. *J Clin Endocrinol Metab*. 2006;**91**(11):4287–4294.
19. Schwimmer JB, Pardee PE, Lavine JE, Blumkin AK, Cook S. Cardiovascular risk factors and the metabolic syndrome in pediatric nonalcoholic fatty liver disease. *Circulation*. 2008;**118**(3):277–283.
20. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care*. 2009; **32**(Suppl 1):S62–S67.
21. Schwimmer JB, Dunn W, Norman GJ, Pardee PE, Middleton MS, Kerkar N, Sirlin CB. SAFETY study: alanine aminotransferase cutoff values are set too high for reliable detection of pediatric chronic liver disease. *Gastroenterology*. 2010;**138**(4):1357–1364e2.
22. Chan WK, Sthaneshwar P, Nik Mustapha NR, Mahadeva S. Limited utility of plasma M30 in discriminating non-alcoholic steatohepatitis from steatosis: a comparison with routine biochemical markers. *PLoS One*. 2014;**9**(9):e105903.
23. Bacha F, Edmundowicz D, Sutton-Tyrell K, Lee S, Tfayli H, Arslanian SA. Coronary artery calcification in obese youth: what are the phenotypic and metabolic determinants? *Diabetes Care*. 2014;**37**(9): 2632–2639.
24. Bartz SK, Caldas MC, Tomsa A, Krishnamurthy R, Bacha F. Urine albumin-to-creatinine ratio: a marker of early endothelial dysfunction in youth. *J Clin Endocrinol Metab*. 2015;**100**(9):3393–3399.
25. Bottomley PA. Spatial localization in NMR spectroscopy in vivo. *Ann N Y Acad Sci*. 1987;**508**:333–348.
26. Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn Reson Med*. 1993;**30**(6):672–679.
27. Bonetti PO, Pumper GM, Higano ST, Holmes DR Jr, Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *J Am Coll Cardiol*. 2004;**44**(11):2137–2141.
28. Heffernan KS, Patvardhan EA, Kapur NK, Karas RH, Kuvin JT. Peripheral augmentation index as a biomarker of vascular aging: an invasive hemodynamics approach. *Eur J Appl Physiol*. 2012;**112**(8):2871–2879.
29. Tomsa A, Klinepeter Bartz S, Krishnamurthy R, Krishnamurthy R, Bacha F. Endothelial function in youth: a biomarker modulated by adiposity-related insulin resistance. *J Pediatr*. 2016;**178**:171–177 Epub20160823.
30. Bacha F, Lee S, Gungor N, Arslanian SA. From pre-diabetes to type 2 diabetes in obese youth: pathophysiological characteristics along the spectrum of glucose dysregulation. *Diabetes Care*. 2010; **33**(10):2225–2231.
31. Bacha F, Saad R, Gungor N, Arslanian SA. Adiponectin in youth: relationship to visceral adiposity, insulin sensitivity, and beta-cell function. *Diabetes Care*. 2004;**27**(2):547–552.
32. Fabbrini E, Magkos F, Conte C, Mittendorfer B, Patterson BW, Okunade AL, Klein S. Validation of a novel index to assess insulin resistance of adipose tissue lipolytic activity in obese subjects. *J Lipid Res*. 2012;**53**(2):321–324.
33. Targher G, Bertolini L, Padovani R, Rodella S, Zoppini G, Zenari L, Cigolini M, Falezza G, Arcaro G. Relations between carotid artery wall thickness and liver histology in subjects with nonalcoholic fatty liver disease. *Diabetes Care*. 2006;**29**(6):1325–1330.

34. Jin R, Le NA, Cleeton R, Sun X, Cruz Muños J, Otvos J, Vos MB. Amount of hepatic fat predicts cardiovascular risk independent of insulin resistance among Hispanic-American adolescents. *Lipids Health Dis*. 2015;**14**:39.
35. Pacifico L, Anania C, Martino F, Cantisani V, Pascone R, Marcantonio A, Chiesa C. Functional and morphological vascular changes in pediatric nonalcoholic fatty liver disease. *Hepatology*. 2010;**52**(5): 1643–1651.
36. Koot BG, de Groot E, van der Baan-Slootweg OH, Bohte AE, Nederveen AJ, Jansen PL, Stoker J, Benninga MA. Nonalcoholic fatty liver disease and cardiovascular risk in children with obesity. *Obesity (Silver Spring)*. 2015;**23**(6):1239–1243.
37. Huang RC, Beilin LJ, Ayonrinde O, Mori TA, Olynyk JK, Burrows S, Hands B, Adams LA. Importance of cardiometabolic risk factors in the association between nonalcoholic fatty liver disease and arterial stiffness in adolescents. *Hepatology*. 2013;**58**(4):1306–1314.
38. Lomonaco R, Bril F, Portillo-Sanchez P, Ortiz-Lopez C, Orsak B, Biernacki D, Lo M, Suman A, Weber MH, Cusi K. Metabolic impact of nonalcoholic steatohepatitis in obese patients with type 2 diabetes. *Diabetes Care*. 2016;**39**(4):632–638.
39. Lee S, Rivera-Vega M, Alsayed HM, Boesch C, Libman I. Metabolic inflexibility and insulin resistance in obese adolescents with non-alcoholic fatty liver disease. *Pediatr Diabetes*. 2015;**16**(3):211–218.
40. Ono M, Okamoto N, Saibara T. The latest idea in NAFLD/NASH pathogenesis. *Clin J Gastroenterol*. 2010;**3**(6):263–270.
41. Pacifico L, Di Martino M, De Merulis A, Bezzi M, Osborn JF, Catalano C, Chiesa C. Left ventricular dysfunction in obese children and adolescents with nonalcoholic fatty liver disease. *Hepatology*. 2014; **59**(2):461–470.
42. Wicklow BA, Wittmeier KD, MacIntosh AC, Sellers EA, Ryner L, Serrai H, Dean HJ, McGavock JM. Metabolic consequences of hepatic steatosis in overweight and obese adolescents. *Diabetes Care*. 2012; **35**(4):905–910.