Original Article
PEDIATRIC OBESITY

Obesity

Macrophages and Fibrosis in Adipose Tissue are Linked to Liver Damage and Metabolic Risk in Obese Children

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Objective: Obesity in childhood is associated with an inflammatory state in adipose tissue and liver, which elevates risk for diabetes and liver disease. No prior study has examined associations between pathologies occurring in adipose tissue and liver to identify elements of tissue damage associated with type 2 diabetes risk. This study sought to determine whether inflammation and fibrosis in abdominal subcutaneous adipose tissue (SAT) in obese/overweight children (BMI-*z* 2.3 ± 0.76) was related to the extent of observed liver disease or type 2 diabetes risk.

Methods: Biopsy samples of abdominal (SAT) and liver were simultaneously collected from 33 Italian children (mean BMI 28.1 ± 5.1 kg/m^2 and mean age 11.6 ± 2.2 years) with confirmed NAFLD. Histology and immunohistochemistry were conducted on biopsies to assess inflammation and fibrosis in adipose tissue and fibrosis and inflammation in liver.

Results: Presence vs. absence of crown-like structures (CLS) in SAT was significantly related to liver fibrosis scores (1.7 ± 0.7 vs. 1.2 ± 0.7, $P = 0.04$) independent of BMI. SAT fibrosis was significantly correlated with a lower disposition index ($r = -0.48$, $P = 0.006$). No other adipose measures were associated with liver disease parameters.

Conclusion: Markers of subcutaneous white adipose tissue inflammation are associated with greater extent of liver fibrosis independent of obesity and SAT fibrosis may contribute to diabetes risk through reduced insulin secretion.

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Introduction

Obesity is marked by excess storage of lipids and a chronic pro-inflammatory state in adipocytes (1). The obesity-associated low-grade inflammation is sustained by the increase of macrophages in adipose tissue (2) and is strongly associated with increased ectopic fat accumulation in the liver (3), which can lead to non-alcoholic fatty liver disease (NAFLD), a process that begins early in life (4). Pediatric obesity prevalence continues to rise internationally (5) and is a strong risk factor for NAFLD, which is now the primary cause of liver disease in children (6) and is increasing in prevalence in the US (7). Adipose and liver tissues demonstrate similar yet independent relationships between excess fat deposition and the ensuing cell-signaling responses and resultant inflammation; however there is very little research that examines how inflammation in both adipose tissue (8) and liver (9) may be linked in children. This is surprising given that the prevalence of NAFLD amongst obese children can be as high as 70% in the United States (10) and even at an early age some children may progress to NASH, marked by the presence of liver fibrosis and inflammation (4).

Increases in adipose tissue have been linked to metabolic dysregulation (11) and increased ectopic lipid deposition in the liver promotes pro-inflammatory mediators (12) that are thought to activate specific liver-resident macrophages known as Kupffer cells, which may mediate the progression from NAFLD to NASH (13) by inducing hepatic injury and fibrosis (14). Similarly, amongst adipocytes, a

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pro-inflammatory state perturbs normal extracellular matrix (ECM) remodeling and favors fibrous collagen deposition (15) that can result in insulin resistance (16) and a diminished capacity for fat mass expansion (17). This inability of adipose fat mass to expand under fibrotic conditions may force ectopic deposition of fat into other organs such as the liver (18), which suggests a potential link between inflammation in adipose and hepatic tissues, yet the specific role of markers of subcutaneous white adipose tissue (WAT) inflammation in metabolic or liver disease is unclear. In support of this potential link, the Scherer group has recently proposed that abnormal adipose tissue, marked by the presence of fibrosis, may be involved in several pathological changes to tissue locally and at a systems level (19) including impaired glucose homeostasis and ectopic fat deposition.

In order to investigate potential links between obesity-induced adipose inflammation and fibrosis and liver disease in children, we conducted immunohistochemical analyses on biopsied tissue from both subcutaneous adipose tissue (SAT) depots and liver. The aim of this analysis was to determine and characterize the extent of inflammation and fibrosis in the adipose tissue of overweight and obese children and examine their relationship with liver disease and type 2 diabetes risk.

Methods

Patients

A total of 40 consecutive patients with biopsy-proven NAFLD seen at Bambino Gesù Children's Hospital were prospectively included in the study from January 2011 to December 2012, and represented a subset of a population previously reported on (9). The study was approved by the Ethics Committee of the Bambino Gesù Children's Hospital and Research Institute in Rome, Italy and all participants signed approved informed consent or assent documents. Inclusion criteria and exclusion criteria were the same as previously reported (9). Autoimmune liver disease, metabolic liver disease, Wilson's disease, celiac disease, and alpha-1-antitrypsin deficiency were ruled out using standard clinical, laboratory, and histological criteria. Adipose samples from 7 of the 40 children biopsied were not viable and these participants were excluded from formal analysis. Complete data was obtained in the remaining 33 participants.

Anthropometric measures

Weight and height were measured using standard procedures. Body mass index (BMI) (kg/m^2) and its standard deviation score (Z score) were calculated using US reference data (20,21). Waist circumference (WC) was measured at the highest point of the iliac crest.

Laboratory assessment

Alanine and aspartate transferases (ALT and AST, respectively), gamma-glutamyl-transpeptidase, total triglycerides, and total low-density (LDL) and high-density lipoprotein (HDL) cholesterol were evaluated using standard laboratory methods. Plasma insulin was measured using a radioimmunoassay (Myria Technogenetics, Milan, Italy). All participants underwent a standard oral glucose tolerance test (OGTT) performed with 1.75 g of glucose per kilogram of body weight (up to 75 g), and glucose and insulin were measured at 0, 30, 60, 90, and 120 min. The degree of insulin sensitivity/resistance was determined via the homeostatic model assessment (HOMA) (22) and by the OGTT-derived insulin sensitivity index (ISI) (23). Both the HOMA and the OGTT-derived ISI have a significant correlation with the gold standard euglycemic hyperinsulinemic glucose clamp technique (23). A HOMA value >2 or ISI value <6 were considered an indication of insulin resistance.

Pro-inflammatory markers and adipocytokines

Serum C-reactive protein (CRP) was determined via a high sensitivity latex agglutination method on HITACHI 911 Analyser (Sentinel Ch., Milan). The kit had a minimum detection of less than 0.05 mg/l, and a measurable concentration range up to 160 mg/l. The intra-assay and inter-assay variation coefficients were, respectively, 0.8-1.3 and 1.0–1.5%. Serum tumor necrosis factor (TNF)-α and interleukin (IL)−6 were measured by sandwich ELISA (R&D System Europe Ltd, Abingdon, UK). For TNF-α, the kit had a sensitivity of 0.12 pg/ml in a 200-μL sample size and a range of 0.5–32 pg/ml. The intra- and inter-assay coefficients of variation were 5.9% and 12.6%, respectively. For IL-6, the kit had a sensitivity of 0.25 pg/ml in a 50-μl sample size and a range of 3.9–250 ng/ml. The intra- and inter-assay coefficients of variation were 3.4% and 5.8%, respectively. Serum adiponectin was measured by ELISA kit according to the manufacturer's protocol (Ray Biotech, Norcross, GA)

Liver histology

The clinical indication for biopsy was either to assess the presence of NASH and degree of fibrosis and/or to rule out potential other liver diseases. Liver biopsy was performed in all children after an overnight fast, using an automatic core biopsy 18 gauge needle (Biopince, Amedic, Sweden) under general anesthesia and ultrasound guidance. A Sonoline Omnia ultrasound machine (Siemens, Munich, Germany) equipped with a 5-MHz probe (5.0 C 50, Siemens) and a biopsy adaptor were employed. The length of liver specimen was recorded and only samples with a length $\geq$15 mm and including at least 5–6 complete portal tracts were considered adequate for the purpose of the study. It is worth noting that we have previously shown that a high prevalence of necro-inflammatory fibrosis can be found in children with ultrasonographic evidence of steatosis yet normal ALT levels at the time of biopsy, with 81% of patients with normal ALT presenting with fibrosis (24). Therefore, liver biopsies were conducted even in some patients with normal levels of ALT. Additionally, the use of an approach that allowed for the obtainment of a fat biopsy at the time of liver biopsy through implementing an additional pass of the biopsy needle minimized any additional risk to the patient. Biopsies were routinely processed (i.e., formalin-fixed and paraffin-embedded) and sections of liver tissue were stained with hematoxylin–eosin, Van Gieson, Periodic acid–Schiff diastase, and Prussian blue stain. Biopsies were evaluated by a single hepatopathologist who was blinded to clinical and laboratory data. Steatosis, inflammation, hepatocyte ballooning, and fibrosis were scored using the NAFLD Clinical Research Network (CRN) criteria (25): Briefly, steatosis was graded on a 4-point scale: grade 0 = steatosis involving $<$5% of hepatocytes; grade 1 = steatosis involving up to 33% of hepatocytes; grade 2 = steatosis involving 33–66% of hepatocytes; and grade 3 = steatosis involving $>$66% of hepatocytes. Lobular inflammation was graded on a 4-point scale: grade 0 = no foci; grade 1 = $<$2 foci per $\times$200 field; grade 2 = 2–4 foci per $\times$200 field; and grade 3 = $>$4 foci per $\times$200 field. Hepatocyte ballooning was graded from 0 to 2:

TABLE 1 Subject characteristics

	All ($n = 33$)	Lean ($n = 7$)	Overweight ($n = 16$)	Obese ($n = 10$)
Population characteristics				
Age (years)	11.6 (2.2)	10.8 (2.2)	11.6 (2.1)	12.4 (2.5)
Gender (M:F)	20/13	4/3	11/5	5/5
BMI (kg/m^2)	28.1 (5.1)	21.8 (2.2)	27 (1.1)	34.2 (3.8)
BMI *z*-score	2.3 (.76)	1.8 (.42)	2.6 (.99)	2.4 (.28)
WC (cm)	89.3 (8.5)	83.3 (11.8)	89.9 (7.8)	92.6 (4.8)
Biochemical measures				
Fasting plasma glucose (mg/dl)	80.2 (13)	80.7 (7.6)	80 (16.8)	80.3 (9.7)
Fasting plasma insulin (μU/l)	16.7 (11.2)	13.3 (9.6)	14.54 (7)	22.8 (15.4)
Matsuda index	3.9 (3.1)	3.8 (2.1)	3.9 (3.2)	4 (3.8)
HOMA-IR	3.3 (2.3)	2.7 (1.9)	2.8 (1.4)	4.6 (3.3)
Insulinogenic index (IGI)	2.9 (6.4)	2.1 (1.4)	4.2 (9)	4.6 (3.2)
Disposition index (IGI*ISI)	9 (16.5)	6.8 (5.6)	12.9 (23.4)	4.6 (3.6)
AST (IU/l)	29.2 (8.8)	30.3 (6.3)	29.9 (8.6)	27.1 (10.9)
ALT (IU/l)	34.4 (15.6)	45.2 (20.9)	33.1 (14.2)	28.6 (10.3)
GGT (ng/dl)	20.6 (9.2)	19.9 (7.6)	19.9 (11.2)	22.4 (7.4)
Adiponectin (ng/dl)	21.9 (2.3)	21.9 (2.7)	22 (1.9)	21.7 (2.8)
CRP (ng/dl)	1.6 (.4)	1.9 (.4)	1.5 (.4)	1.7 (.4)
IL-6 (ng/dl)	9.5 (4.5)	9.1 (2.2)	8.6 (4.7)	11.1 (5.2)
TNF-α (ng/dl)	6.5 (1.9)	6.7 (1.4)	6.4 (2)	6.5 (2.1)

Values are presented as means with standard deviation. BMI = body mass index, WC = waist circumference, ALT = alanine transferase, AST = aspartate transferase, GGT = gamma-glutamyl-transpeptidase, HOMA-IR = homeostatic model of insulin resistance, CRP = c-reactive protein, TNF-α = tumor necrosis factor alpha, IL-6 = interleukin six.

0 = none; 1= few balloon cells; and 2 =many/prominent balloon cells. The stage of fibrosis was quantified using a 5-point scale: stage 0 = no fibrosis; stage 1 = perisinusoidal or periportal (1a = mild, zone 3, perisinusoidal; 1b = moderate, zone 3, perisinusoidal; 1c = portal/periportal); stage 2 =perisinusoidal and portal/periportal; stage 3 = bringing; and stage 4 = cirrhosis.

Features of steatosis, lobular inflammation, and hepatocyte ballooning were combined to obtain the NAFLD activity score (NAS). As recently recommended by the NASH Clinical Research Network (25), a microscopic diagnosis based on overall injury pattern (steatosis, hepatocyte ballooning, inflammation) as well as the presence of additional lesions (e.g., zonality of lesions, portal inflammation and fibrosis) was assigned to each case. Accordingly, biopsies were subdivided into: not-NASH and definite NASH subcategories (26).

Adipose histology

Following the liver biopsy, a second pass with the same needle through the same insertion point was used to obtain an abdominal SAT biopsy in all children. Biopsies were routinely processed (i.e., formalin-fixed and paraffin-embedded) and sections of adipose tissue were stained with hematoxylin–eosin and CD68 antibody (Leica Biosystems, Newcastle, UK) or picrosirius red. The CD68 antibody incubation time was 15 min and antigen retrieval was at pH 8 for 20 min on the Leica bond. Biopsies were evaluated by a single technician who was blinded to clinical and laboratory data. For the CD68 stained sections, four consecutive 5-micron sections were obtained for each subject. Two independent fields at ×20 magnification were captured for each section, for a total of eight images per subject. Adipose cell size (micron^2), adipose cell count, isolated macrophages, and crown-like structure (CLS) counts were obtained for each field captured using Fiji quantitative microscopy software (27). For each field, isolated macrophage per cm^2 and CLS per cm^2 were calculated by dividing by the area of the field measured. For each subject, the mean values for adipose cell size, macrophages per cm^2, and CLS cm^2 were obtained. For the collagen analysis, three non-serial 5 micron sections were obtained for each subject and stained with picrosirius red and prepared according to Bedossa et al. (28). Two independent fields at ×20 magnification were captured for each section. Fiji software (27) was used to obtain total collagen area and a collagen to adipose ratio.

Data analysis

Values are expressed mean ± standard deviation. Variables were assessed for normality and non-normal data was log (ln) transformed for analyses. Wilcoxon test was used to compare between-group differences. Non-parametric Spearman's correlations were used to examine correlations and partial correlations were conducted controlling for age, gender, and BMI as covariates. Statistical significance was set at $P < 0.05$. All analyses were performed on SPSS v18 (SPSS Inc, Chicago IL).

Results

Anthropometric associations with SAT and liver tissue measures

Complete measures were obtained in 33 of the 40 participating children. Anthropometric characteristics are reported in Table 1, together

TABLE 2 Adipose and liver immunohistological measures

	All ($n = 33$)	Lean ($n = 7$)	Overweight ($n = 16$)	Obese ($n = 10$)
Adipose histology				
Macrophage count	4.9 (3.6)	4.7 (2.2)	4.9 (4.1)	5.1 (3.9)
Macrophages per cm^2	0.51 (0.35)	0.78 (0.52)	0.48 (0.25)	0.37 (0.28)
CLS (−/+)	19/14	6/1	10/6	3/7
CLS per cm^2 (among CLS+)	0.06 (0.05)	0.07 (NA)	0.07 (0.06)	0.05 (0.03)
Adipose cell area (μ^2)	2267.1 (704.4)	1924.2 (364.9)	2148.1 (648)	2742 (800.8)
Collagen ratio	21.1 (14.5)	25.1 (10.8)	22.1 (15.6)	16.7 (15.1)
Liver histology frequency (%)				
Fibrosis				
0–1	17 (60.6)	5 (71.4)	8 (50)	7 (70)
2–3	13 (39.4)	2 (28.6)	8 (50)	3 (30)
Steatosis				
0–1	25 (75.7)	2 (28.6)	3 (18.8)	2 (20)
≥2	8 (24.2)	5 (71.4)	13 (81.2)	8 (80)
Lobular inflammation				
0–1	15 (44.5)	3 (42.9)	9 (56.2)	3 (30)
2	18 (54.5)	4 (57.1)	7 (43.8)	7 (70)
Portal inflammation				
0–1	19 (57.5)	3 (42.9)	10 (62.5)	6 (60)
2	14 (42.4)	4 (57.1)	6 (37.5)	4 (40)
Ballooning				
0–1	23 (69.7)	5 (71.4)	12 (75)	6 (60)
2	10 (30.3)	2 (28.6)	4 (25)	4 (40)
NAS				
0–2	1 (3)	1 (14.3)	0 (0)	0 (0)
3–4	15 (45.5)	2 (28.6)	9 (56)	4 (40)
≥5 (NASH)	17 (51.5)	4 (57.2)	7 (44)	6 (60)

Values are presented as means with standard deviation, unless otherwise noted. NASH = non alcoholic steatohepatitis, CLS = crown-like structure, NAS = NAFLD activity score.

with a panel of adipocytokines and additional subject descriptors. Mean BMI was 28.1 ± 5.1 kg/m^2 with a mean BMI *Z*-score of 2.3 ± 0.76 (lean, $n = 7$; overweight, $n = 16$, obese, $n = 10$). Twenty of the 33 children were male and the mean age was 11.6 ± 2.2 years.

Overweight/obese children had a higher mean adipocyte area than the lean participants (2742 ± 800.8 cm^2 vs 1924.2 ± 364.9 cm^2; $P = 0.03$, respectively) and quantity of CLS per cm^2 in adipose tissue was positively correlated with BMI and WC in all subjects ($r = 0.43$, $P = 0.01$ and $r = 0.41$, $P = 0.01$, respectively). Fasting plasma insulin and HOMA-IR values increased from lean to overweight to obese, however significantly higher fasting plasma insulin (22.8 ± 15.4 vs.14.2 ± 7.7 μU/l; $P = 0.03$) and HOMA-IR (4.6 ± 3.3 vs. 2.8 ± 1.5; $P = 0.03$) were only observed in obese vs. overweight participants. There were no significant differences in any other insulin or glucose parameters by obesity status.

Mean scores for liver steatosis (1.8 ± 0.6 vs. 2.0 ± 0.6 vs. 2.2 ± 0.7), ballooning (1.1 ± 0.6 vs. 1.2 ± 0.5 vs. 1.5 ± 0.6), and NAS (4.4 ± 1.5 vs. 4.6 ± 1.2 vs. 5.2 ± 1.3) increased as a function of obesity (lean vs. overweight vs. obese, respectively), but these trends did not reach significance. Measures of liver disease were not associated with any glucose or insulin parameters.

Subcutaneous WAT morphology

Of the 33 participants, 14 had one or more CLS present in SAT sections (Table 2). Microscopy identified clear clinical differences in adipocyte cell size, CLS presence, and fibrosis by obesity classification (Figure 1). Adipose cell area was significantly positively associated quantity of CLS/cm^2 ($r = 0.4$, $P = 0.01$). Obese participants exhibited less SAT collagen than their overweight or lean counterparts (16.7 ± 15.1 vs. 22.1 ± 15.6 vs. 25.0 ± 10.8, respectively), however these values did not significantly differ.

Subcutaneous WAT and liver disease

A NAS score of ≥5 was present in over 50% of the study participants (Table 2) and histology (18) confirmed NASH with advanced steatosis and fibrosis (Figure 2). Anthropometric and biochemical measures were not significantly different when means were compared by NAS category (0–4 and ≥5). Serum pro-inflammatory cytokines (CRP, IL-6, and TNF-α) were not related to adipose inflammation and fibrosis or NAFLD features in this cohort. Serum adiponectin was not related to any aspects of adiposity or markers of subcutaneous WAT inflammation, however mean serum adiponectin was significantly lower in the high-steatosis scored participants ($P_{\text{trend}} < 0.001$) and displayed a highly significant negative

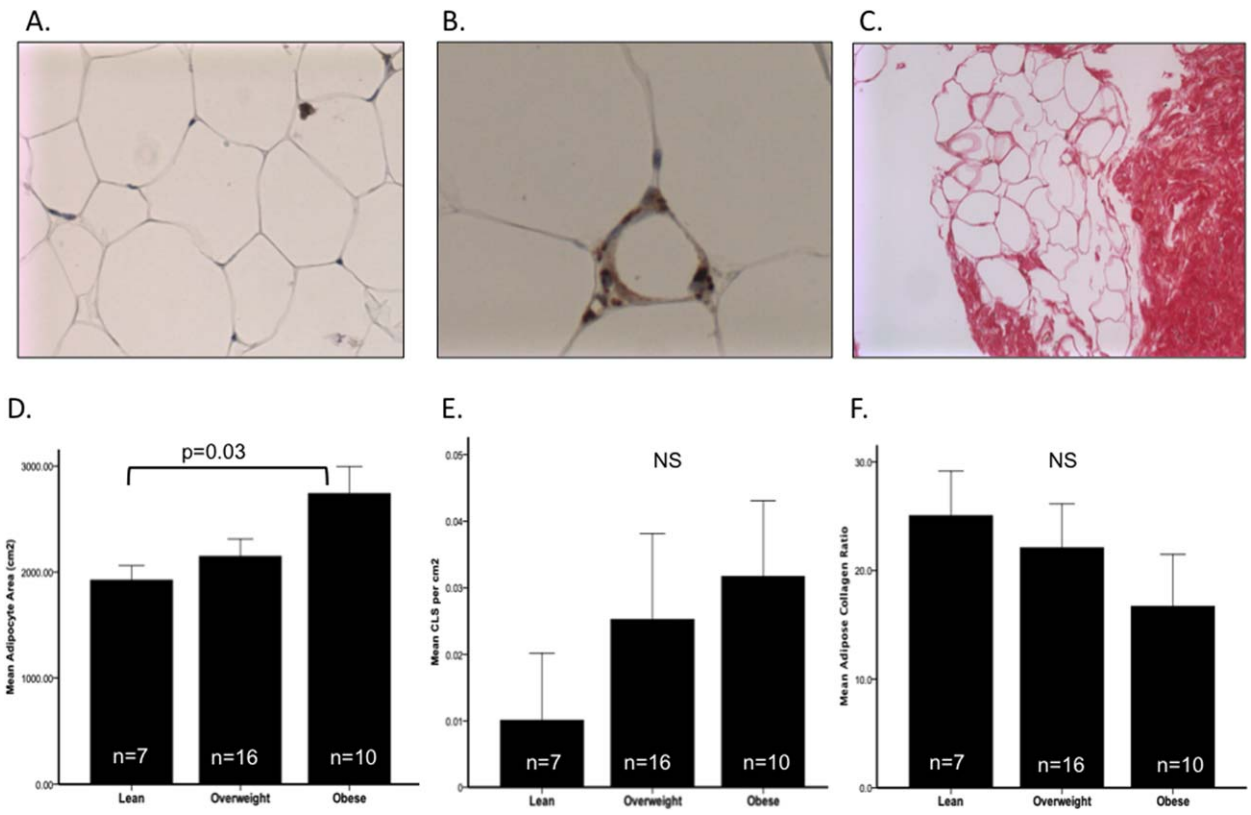

Figure 1 Adipose cell size, CLS presence, and collagen ratio as a function of obesity. Histological analysis of SAT adipose sections (Panel A) at 20× magnification demonstrated a significant increase in adipocyte cell area (μ^2) between lean and obese participants (Panel D). CD68 immunohistochemical staining for macrophages identified CLS in 14 of 33 participants (Panel B) and a clinical increase in CLS per cm^2 by obesity was observed (Panel E). Picrosirius red staining was used to assess fibrosis in SAT through obtaining a collagen ratio (Panel C). A trend in decreasing collagen ratio as a function of obesity was observed (Panel F). CLS = crown-like structures, SAT = subcutaneous adipose tissue. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

correlation with NAS score ($r = -0.62$, $P < 0.001$) even when controlling for BMI z-score and gender (-0.31, $P = 0.05$). Subjects who had SAT with CLS demonstrated significantly higher liver fibrosis scores (CLS+; 1.7 ± 0.7 vs. CLS−; 1.2 ± 0.7, $P = 0.04$), independent of BMI. Similarly, CLS+ participants had a tendency for greater hepatocyte ballooning (1.4 ± 0.7 vs. 1.0 ± 0.5, $P = 0.07$) compared to participants with no CLS. There were no other statistically significant relationships between indicators of SAT inflammation or fibrosis by NASH status.

Subcutaneous WAT and glucose homeostasis

Adipose collagen accumulation was significantly inversely correlated with insulinogenic index ($r = -0.37$, $P = 0.03$) and disposition index (DI) ($r = -0.48$, $p = 0.006$), but not with HOMA or fasting plasma insulin or glucose. The relationship between collagen and DI was maintained ($r = -0.31$, $P = 0.05$) after adjusting for age, gender, and BMI (Figure 3).

Discussion

This is the first study to examine the links between adipose tissue inflammation/fibrosis and liver damage in children with biopsy-proven NAFLD. Although this is a preliminary study in a small sample, we identified several novel findings that merit further investigation: 1) Markers of subcutaneous WAT inflammation were significantly related to liver fibrosis; 2) Fibrosis in SAT was significantly associated with impaired DI, which was supported by clinically higher glucose area under the curve and lower insulin area under the curve values observed in patients with higher adipose fibrosis; 3) Obesity was significantly associated with markers of subcutaneous WAT inflammation and obese children tended to have higher qualitative liver disease scores than lean counterparts. These data suggest that increasing obesity is strongly related to both adipose tissue damage and liver damage with some evidence of a link between inflammatory CLS presence in SAT and liver disease.

Our study has established a novel link between subcutaneous adipose inflammation, as indicated by presence of CLS, and the extent of liver fibrosis in children. Nearly half of the children in the present study had CLS in adipose tissue and the number of CLS was positively related to obesity status and adipose cell size. This is consistent with a prior study (17) in which the number of CLS increases with adipocyte hypertrophy, indicating a heightened inflammatory state. However, a recent study in children did not detect CLS in the abdominal adipose tissue of healthy, overweight children (8).

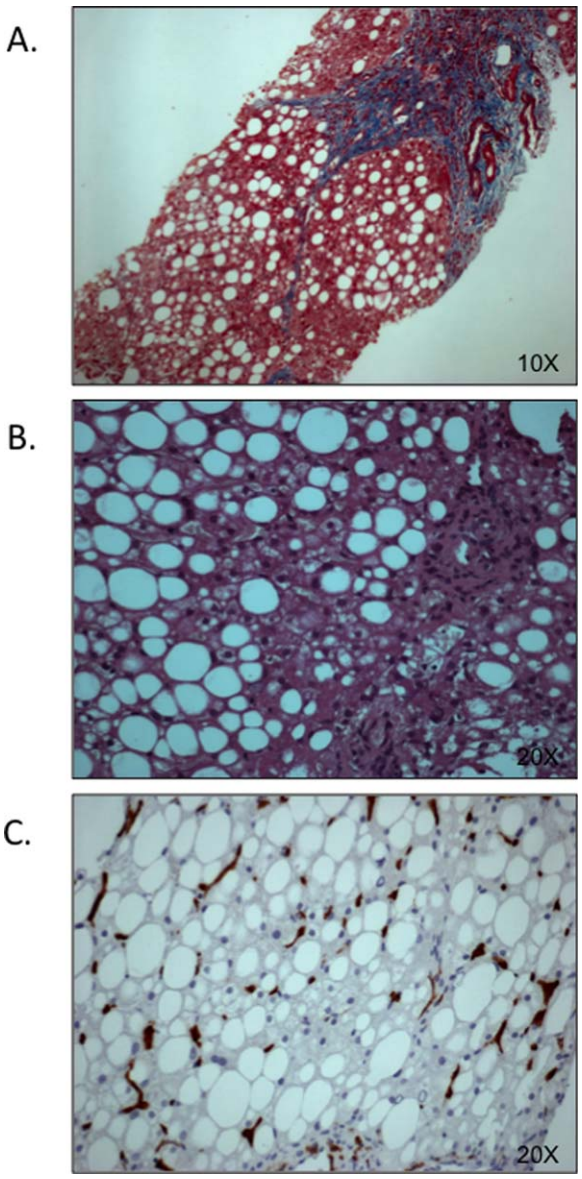

Figure 2 Liver histology: steatosis, fibrosis, and inflammation. Panel A (10× magnification) is a representative image of NASH with steatosis, and fibrosis score F3. Panel B represents a child with NASH (steatosis grade 2, ballooning grade 1, and inflammation grade 1). Panel C depicts steatosis score 2 and hepatic inflammation as evidenced by CD163+ Kupfer cell staining. Images adapted from Alkhouri et al. (9). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

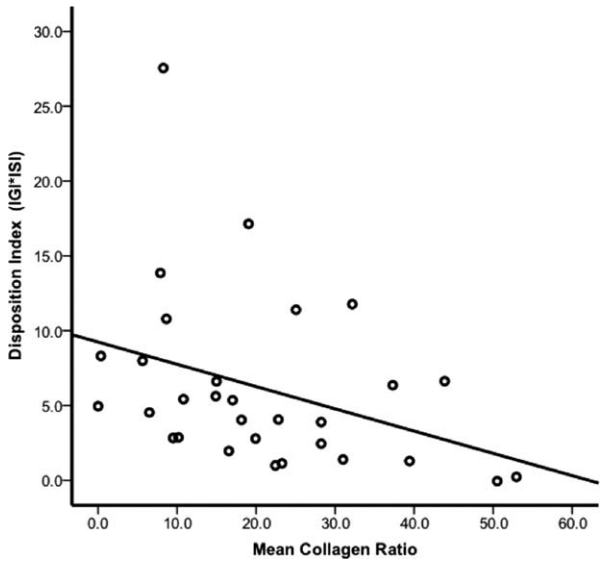

Figure 3 Mean adipose collagen ratio is inversely associated with disposition index. Mean adipose collagen ratio is displayed on the *x*-axis and mean disposition index is on the *y*-axis. Disposition index decreased as a function of increasing adipose fibrosis. Graphical data are presented as raw, unadjusted values and the correlation (−0.31, $P = 0.05$) persisted after controlling for age, gender, and BMI.

Tordjman et al. (29) recently demonstrated that there are no observable differences in macrophage number or CD68 gene expression in SAT by NASH status in adults; however they were able to show highly significant differences by liver disease status in both of these measures when examining deep SAT or VAT, suggesting differential roles for distinct adipose depots on liver disease. Although we were not able to determine whether our adipose samples represented deep SAT vs. SAT, the abundance of CLS is consistent with other work in deep SAT tissue (29). Increased delivery of pro-inflammatory factors from macrophages to the liver along with free fatty acid flux may be one possible link between VAT and liver disease (30), but this observation has yet to be demonstrated in SAT depots (29). Given that CLS+ subjects in our study had significantly greater liver fibrosis scores, our data suggests that markers of subcutaneous WAT inflammation may play a role in modulating inflammation in the liver possibly through an increase in pro-inflammatory factors in the liver. Interestingly, in a study composed of obese adults, subjects with CLS in their SAT had elevated liver fat, as measured by proton magnetic resonance spectroscopy (31), and similar to our study there was no association between adipocyte cell size and steatosis. It is possible that quantitative measures of liver disease, such as liver fat fraction, would demonstrate a stronger relationship to inflammatory SAT measures in children and other adipose regions may be linked to liver disease. We were not able to test these hypotheses directly, as qualitative measures of liver fat were not available, however subsequent studies should include more comprehensive measures of steatosis.

Adipose tissue fibrosis, as indicated by SAT collagen amount, was inversely related to DI, which is the ability to compensate for insulin resistance through increased insulin secretion. Fibrosis and inflammation in adipose tissue have been previously associated with insulin resistance (32) and although SAT fibrosis did not correlate significantly with any other measures of glucose and insulin dynamics in our population, an observation also noted in adults (33), our participants with greater fibrosis had a clinically higher glucose area under the curve and lower insulin area under the curve in response to an OGTT. Higher collagen deposition in adipose tissue is thought to inhibit adipose expansion, which can lead to inflammation through the recruitment of macrophages, pointing to a potential relationship between adipose ECM and inflammation (8,19). Interestingly, a recent study in adults found obese subjects had a higher degree of pericellular adipose fibrosis in both SAT and omental adipose tissue than lean participants (32) with no significant difference in total fibrosis by obesity status. Similarly, in healthy children,

obese individuals were found to have significantly less collagen that lean peers and this collagen was inversely associated with both adipose cell size (8). Our findings are consistent with this observation, yet it remains unclear whether the amount of fibrosis in the adipose tissue of children is related to a pathology or if it reflects the normal physiological development and growth of adipose tissue (8). This suggests that collagen type as well as adipose region may contribute differentially to the regulation of glucose homeostasis. Thus, it remains possible that the relationships between specific adipose depots, the types of collagen in said depots and glucose homeostasis are very different and modulated to a greater extent by the degree of inflammation in the developing adipose tissues of children than that of adults.

Several important limitations of this study should be mentioned. Our relatively small sample size was useful in identifying preliminary relationships between features of inflamed adipose morphology and liver disease for future exploration, but the small size limited us from finding other expected associations between adipose and liver inflammation. This initial study was limited to Italian children and although some findings have been reported in some studies in children and adults, the links between adipose tissue and liver tissue may vary based on age, maturation status, and ethnicity. In this initial study, we did not quantify SAT or visceral adipose tissue and our measures of adipose tissue fibrosis were restricted to total rather than subtypes of collagen, which limits the application of our findings to general relationships between SAT and total collagen with liver disease. The surgical biopsy of adipose tissue, when compared to needle-biopsy, is an ideal sampling technique (34). However, because the children in this study were subject to a liver biopsy, we chose to minimize further risk and ethical burden and perform a needle biopsy. Our measures of insulin resistance and DI were based on data from an OGTT rather than intravenous glucose tolerance tests or clamp methods and we did not assay c-peptide, which could be used to further verify insulin resistance, beyond DI, and test for associations with SAT collagen. Despite these limitations, the results of this preliminary study identify novel findings that merit further and more detailed studies that address the present limitations.

In summary, our study has demonstrated a link between adipose tissue inflammation and liver fibrosis, suggesting a role for markers of subcutaneous WAT inflammation in the mediation of liver inflammation. Additionally, higher levels of SAT collagen appear to effect glucose homeostasis indicating that SAT inflammation and fibrosis could be risk factors for insulin resistance and type 2 diabetes. Obesity clearly negatively impacts SAT health in children while increasing risk for NAFLD and NASH. Inflammation and fibrosis in SAT may be involved in promoting hepatic fibrosis and insulin resistance in children with NAFLD, and these relationships require further investigation. O

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