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ABSTRACT

Severe obesity (SO) affects about 6% of youth in US, augmenting the risks for cardiovascular disease and Type 2 diabetes.

Herein, we obtained paired omental (omVAT) and abdominal subcutaneous (SAT) adipose tissue biopsies from obese girls with SO, undergoing sleeve gastrectomy (SG), to test whether differences in cellular and transcriptomic profiles between omVAT and SAT depots affect insulin sensitivity differentially. Following weight loss, these analyses were repeated in a subgroup of subjects having a second SAT biopsy.

We found that omVAT displayed smaller adipocytes compared to SAT, increased lipolysis through adipose triglyceride lipase (ATGL) phosphorylation, reduced inflammation and increased expression of browning/beige markers. Contrary to omVAT, SAT adipocyte diameter correlated with insulin resistance. Following SG, both weight and insulin sensitivity improved markedly in all subjects. SAT adipocytes size became smaller showing an increased lipolysis through perilipin-1 phosphorylation, decreased inflammation and increased expression in browning/beige markers.

In summary, in adolescent girls with SO, both omVAT and SAT depots showed distinct cellular and transcriptomic profiles. Following weight loss, the SAT depot changed its cellular morphology and transcriptomic profiles into a more favorable one. These changes in the SAT depot may play a fundamental role in the resolution of insulin resistance.
INTRODUCTION

Severe obesity is the fastest-growing subcategory of obesity in youth afflicting ~ 6% of all youth in the United States (1-7). Of note, tracking of adiposity from childhood into adulthood is much stronger in the severely obese (8-10), with a high body mass index (BMI) in adolescence being associated with increased risk of cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and premature death (11-16).

Given the limited effectiveness of lifestyle and pharmacological interventions for severe obese in youth, surgical procedures that have proven health benefits for adults are increasingly being considered for severely obese adolescents (1, 17). Recently, Inge et al provided longitudinal five-year outcomes of gastric bypass in adolescents as compared with adults, indicating that adolescents had remission of diabetes and hypertension more often than adults (18). The outcomes data from the Teen–LABS have greatly reinforced the use of bariatric surgery in adolescents with severe obesity, which is now officially recommended by the Endocrine Society Clinical Practice Guideline (19). Despite the clinical relevance of the Teen-LABS studies, the mechanisms by which weight loss induced by bariatric surgery, specifically by sleeve gastrectomy (SG), leads to greater remission in cardiometabolic health in youth is unknown. It is conceivable that changes in the biology of two key adipose depots, the omental (omVAT) and superficial abdominal (SAT), after weight loss have a greater modulatory effect on metabolism, given the greater plasticity of the white adipose tissue during adolescence. However, the lack of parallel assessment of the omental vs subcutaneous abdominal depots in adolescents
with severe obesity greatly limits the understanding of the putative inter-depot differences and their potential changes following the weight loss after SG.

Lipolysis, the process by which TGs are hydrolyzed to FFAs and glycerol, occurs by sequential action of Adipose Triglyceride Lipase (ATGL), Hormone-Sensitive lipase (HSL), and Monoglyceride Lipase (MGL) (20-26). The first step of the lipolytic cascade involves the activation/phosphorylation of ATGL that appears to be dependent on CGI-58 association (20-23). During this stage, a lipid droplet-associated surface protein Perilipin-1 (PLIN1) elicits an important role during the lipolytic cascade as suppressor of CGI58 (24). When inactivated, PLIN1 binds to CGI58 and blocks the activation of ATGL. When PLIN1 is phosphorylated, it releases CGI58 and therefore the lipolytic cascade can continue. During this first step of lipolysis, TAG is hydrolyzed to diacetylglcerol (DAG) and TG. The second step shows the involvement/phosphorylation of HSL, that uses DAG as substrate and produces monoacylglycerol (MAG) and TG (25). Finally, during the final step of lipolysis, the Monoacylglycerol Lipase (MGL) degrades MAG generating free fatty acid and glycerol (26). Both hormones/nutrients regulate this important pathway. During negative energy balance states, activation of lipolysis results in a profound increase in FFA release from AT, thereby providing the organism with substrates for oxidative metabolism.

To dissect differences in cellular morphology and biologic pathways between the omVAT depot compared to that of the SAT, we obtained paired adipose tissues from these two depots in a group of obese adolescent girls with severe obesity that were undergoing SG. Additionally, we followed these subjects during their weight loss over a period of a year
and in a sub-group we repeated the abdominal SAT biopsy after weight loss. We tested the following hypotheses: 1- differences in the cellular, lipolytic activity and transcriptomic profiles of the SAT compared to that of the omVAT depot may play an essential role in the development of insulin resistance in youth with severe obesity; 2- weight loss after the bariatric surgery and the dramatic metabolic changes occurring in the SAT may reduce insulin resistance and its ominous consequences, 3- and whether weight loss induced by SG might be associated with browning of the SAT depot.
RESULTS

Anthropometric, clinical and metabolic characteristics at baseline.

From “The Yale Study of Body Fat Patterning in Obese Adolescents” cohort (NCT01966627), we studied 10 female subjects (7 adolescents and 3 young adults) with severe obesity (class 2/3 obesity), that underwent bariatric surgery using sleeve gastrectomy (SG) procedure. The anthropometric, clinical and metabolic characteristics of participants are presented in Table 1. All subjects had mean age of 18.3 years (range 16-22), Tanner stage 4 to 5, and a mean BMI of 45.9 kg/m2 (range 36.7-57.2). Among all the participants, there were 3 subjects with early T2DM and 2 with Impaired Glucose Tolerance.

Adipocytes cell size distribution in SAT and omVAT depot.

To determine the difference in adipocytes morphology, we obtained paired samples from both abdominal superficial subcutaneous adipose tissue (SAT) and omental adipose tissue (omVAT) during the SG surgery and fixed the tissues in osmium tetroxide, as previously described (27).

The adipocyte cell size distribution profile from the SAT depot showed a distinct shift to the right compared with that seen in omVAT, with the adipocytes from SAT being significantly larger than those from omVAT (p=0.002) (Figure 1A and Table 2). Interestingly, while the peak diameter of SAT cells directly correlated with the increase in insulin resistance (HOMA-IR) (p=0.037), in the omVAT such correlation was not present
Correlations between BMI and cell peak diameter were not statistically significant neither in SAT nor in omVAT (SAT: $r=-0.21$, $p=0.56$; omVAT: $r=0.06$ $p=0.89$). Table 2 shows additional adipose cell size parameters in SAT and omVAT. Interestingly, the percentage of small cells (i.e. cells below nadir) and the ratio of small to large cells in SAT were significantly higher compared to those from the omVAT depots. Of note, we found a strong positive correlation between omVAT cell peak diameter and both percentage of SAT small cells ($r=0.729$, $p=0.021$) and SAT ratio of small to large cells ($r=0.754$, $p=0.015$) (Figure 1C-D). However, the opposite was not true. These significant correlations suggest a possible cross-talk from omVAT to SAT, but not vice-versa (SAT to omVAT).

**Before Sleeve Gastrectomy, omVAT and SAT depots showed similar profiles for genes regulating lipolysis but distinctly different profiles for browning and inflammation markers.**

To determine potential differential gene signature/profiles in SAT versus omVAT we used RNA-sequencing (RNA-seq). Cluster analysis using 4155 most significantly expressed genes correctly distinguished SAT and omVAT as shown by the heatmap (Figure 2A) and principal component analysis (PCA) (Figure 2B). Furthermore, 1492 differentially expressed transcripts were identified ((false discovery rate) FDR>0.05, $p_{adj}<0.05$), of which 448 were upregulated (Log2 (fold change) > 2, $p_{adj}<0.05$) and 1044 were downregulated genes (Log2 (fold change) < -2, $p_{adj}<0.05$). We elected to focus on genes involved in lipid metabolism, white/browning adipose tissue markers, and inflammation,
based on the degree of differential expression of each gene and its known functional roles in metabolic diseases.

Ingenuity Pathway Analysis (IPA) revealed that the top 20 highly enriched canonical pathways were mainly associated to several transcription factors pathways, stem cells pluripotency, as well as WNT/beta-catenin signaling, immune-cells (both granulocyte and agranulocyte) migration. Surprisingly, IPA pointed out a significant differential regulation of genes associated with the browning of white adipose tissue in SAT compared to omVAT. In particular among all the genes associated to this process, 14% were downregulated and 5% upregulated in SAT (Figure 2C).

Using RNA-seq analysis we found a distinct pattern in gene expression between SAT and omVAT (Figure 2A-B). Surprisingly, we did not observe any significant difference in the expression of the majority of the key genes involved in lipogenesis and lipolysis (Supplemental Figure S1A-C), as well as many of the genes involved in adipogenesis (Supplemental Figure S1E). However, we found a significant increase in leptin (LEP), cEBPb and Phosphoenolpyruvate Carboxykinase 2 (PCK2) gene expression (Supplemental Figure S1D-F). Of particular note, we found in omVAT a significant increased expression in genes associated with the browning of white adipose tissue (28-33) (including PGC1a, UCP1, CIDEA, and EBF2) compared to SAT depot (Supplemental Figure S1F-G). Interestingly, also CD36 (described as a lipid intake channel and positively correlated with adipocytes differentiation) was significantly reduced in omVAT compared to SAT depot (Supplemental Figure S1H).
In addition, we studied potential differential expression of gene regulating inflammation in omVAT and SAT. Overall, omVAT did display a slight reduction in macrophage content by CD68 gene expression, and a consistent significant decrease in several pro-inflammatory genes (including IL6, MCP-1, iNOS, IL-8, CD11c) compared to SAT. However, the profile of anti-inflammatory genes (Supplemental Figure S1J) was not significantly different between the depots.

In order to quantify more precisely the differential expression found by RNA-seq analysis, the expression levels of genes involved in lipid metabolism, browning and inflammation were confirmed and validated by real-time PCR. As already shown by RNA-seq, we did not find significant differences by real-time PCR between SAT and omVAT in the expression of the major lipolytic, lipogenic and adipogenic genes (Figure 3A-C). Only LEP and CD36 were significantly reduced in omVAT compared to SAT (Figure 3B-C).

Interestingly, we observed a slight reduction of CD68 gene expression (a general inflammation marker) as well as of several pro-inflammatory markers (including IL6, MCP1 and TNFa) in omVAT compared to SAT, but no differences were observed among the anti-inflammatory markers (Figure 3D). Using a specific antibody for CD68, we were also able to visualize and localized by IHC the macrophages in both omVAT and SAT (SAT CD68=22.0±4.0 macrophages/100adipocytes; omVAT CD68=14.9±2.9 macrophages/100adipocytes; p value=0.09). These representative images showed a slight reduction of CD68+ cells in omVAT compared to SAT depot (Figure 3E).

Since we found by both IPA and RNA-seq analysis an increased expression in mitochondrial markers and genes associated with the browning of adipose tissue, we focused our attention on these genes. Using real-time PCR we were able to confirm the
increase in gene expression of several genes associated to browning of white adipose tissue (including \textit{UCP1}, \textit{PGC1a}, \textit{CIDEA}, and \textit{EBF2}) and specific mitochondrial genes (including \textit{COX4L1}, \textit{COX7c}, \textit{PCK2}, and Creatine Kinase1 (\textit{CKMT1})) in the omVAT depot (Figure 4A).

Given that the browning/beiging of white adipose tissue in both human and mouse models is modulated by cold exposure and/or winter (34-39), we tested in our subjects if the season could affect the expression of browning/beige markers in SAT. Overall, we did not observe any significant increase in browning/beige gene expression in SAT from patients that underwent biopsy during the cold season (winter/fall) compared to those that underwent SG during the warm season (summer/spring) (5 subjects each group, data not shown). These observations are in line with the observation previously published by Kern P. et al in SAT from an obese population (40).

Of note, omVAT adipocyte cell peak diameter showed a strong inverse correlation with the gene expression of \textit{UCP1} (master regulator of the browning/beige markers), \textit{PGC1a}, and \textit{EBF2} (Figure 4D-F).

Similar correlations were also observed with the mitochondrial gene expression of \textit{PCK2}, \textit{CKMT1} and \textit{COX4L1} (Figure 4G-I). Interestingly, several other genes that failed to achieve the statistically significant difference between SAT and omVAT depot did show a strong and significant inverse correlation between gene expression and adipocyte peak in omVAT (\textit{CITED1}: \( r=-0.770, p=0.013 \); \textit{TBX1}: \( r=-0.867, p=0.002 \); \textit{CKTM2}: \( r=-0.758, p=0.015 \); \textit{COX7c}: \( r=-0.634, p=0.054 \)). Regardless, no correlation was observed between SAT adipocytes peak and gene expression (data not shown). These data indicate that in
omVAT depot the browning of white tissue is associated with adipocyte with small diameter.

Given that we observed a significant induction in browning of omVAT tissue compared to SAT, we investigated in paired SAT and omVAT tissue from different subjects the expression at protein level of UCP1, the major browning/beige marker. As shown in Figure 4H and Supplemental Figure S2 by immunofluorescence, UCP1 staining was strongly increased in omVAT compared to SAT depot (SAT Fluorescence intensity=9.2±1.5 AU; omVAT Fluorescence intensity= 19.8±1.2 AU; p value=0.002), confirming the qPCR data.

**ATGL but not HSL phosphorylation regulates omVAT adipocytes cell size enhancing lipolysis.**

Since omVAT adipocytes showed smaller cell peak diameter compared to SAT adipocytes, but RNA-seq profile data of lipid metabolism regulatory genes were not able to explain this phenomenon, we examined whether differences in the functional/activity at the protein level of two of the master regulators in lipolysis, ATGL and HSL, would emerge between the two depots and whether the phosphorylation of these key proteins would be correlated with adipocyte size. Therefore, we compared the protein expression level of pATGL and pHSL in SAT and omVAT. In particular, we observed a significant increase in pATGL in omVAT compared to SAT (p<0.01), and a trend in increasing HSL phosphorylation (Figure 5A-C). Moreover, surprisingly, pATGL significantly correlated with SAT cell diameter (r=0.742, p=0.042) but not omVAT cell diameter (Figure 5D) and notably, pATGL correlated with HOMA-IR in both SAT and omVAT (Figure 5E). No
significant correlations were observed between pHSL and cells peak diameter or HOMA-IR in both omVAT and SAT (data not shown).

Interestingly, in obese adolescents PLIN1 protein expression was undetectable in both SAT and omVAT depot (data not shown).

**Effects of SG on changes in weight, insulin sensitivity and fatty liver.**

After bariatric surgery only one patient discontinued the follow-up for personal reasons and therefore was not included in the analysis. As expected, all patients who underwent SG had a marked reduction in body weight, absolute BMI and % change in BMI (Table 3), reaching after six months a plateau in body weight loss which was maintained throughout the 10-months follow-up visit (Figure 6A). Interestingly, six out of 9 patients had a repeated measure of insulin sensitivity using the HOMA-IR, which indicated a strong improvement in insulin-sensitivity, particularly in the obese girls that were more insulin resistant (Figure 6B).

During the follow-up period, 4 girls consented to having a second abdominal SAT biopsy, thus providing the unique opportunity to compare the changes in the SAT depot characteristic before (Baseline) and after weight lost (Follow-up biopsy). These 4 subjects were representative of the main group, since they showed a reduction in BMI, % change in BMI and body weight after surgery similar to the other subjects (Table 3 and Supplemental Figure S3A-D).

**SAT adipocytes are smaller after weight loss induced by SG.**
The Multisizer adipose cell size profile from SAT follow-up biopsy showed a clear shift to the left compared to the baseline biopsy cell curve profile. The cell peak calculated for the baseline biopsy (124.86±5.61 μm) was significantly higher than the cell peak calculated from the follow-up biopsy (99.99±0.71 μm, p=0.0046) (Figure 6C-D and S3E-H).

**Gene profiling after weight loss showed an increase in lipolytic, and induction of genes regulating browning activity but a reduction in pro-inflammatory genes in the SAT depot.**

We analyzed using both RNA-seq and real-time PCR the expression of the same panel of genes associated to lipid metabolism, browning and inflammation markers in SAT obtained at baseline and at follow-up biopsy from the same subjects. We found an increase in lipolytic and adipogenic genes (including *ATGL*, *HSL*, *PLIN1*, *PLIN4*, and *ADIPOQ*) expression levels in the follow-up biopsy compared to the baseline biopsy (Figure 7A-C and Supplemental Figure S4A-C).

In contrast, we found a reduction in pro-inflammatory status of the SAT after weight loss as shown by stark downregulation in *IL6*, *MCP1*, *IL8*, *IL1b* and *CD11c* (Figure 7D and Supplemental Figure S4D). Overall, the total amount of macrophages was maintained between baseline and follow-up biopsy, as showed by *CD68* mRNA expression levels (Figure 7D) and immunohistochemistry using a specific CD68 antibody (Baseline biopsy CD68=22.0±4.0 macrophages/100adipocytes; Follow-up biopsy CD68=18.3±2.4 macrophages/100adipocytes; p value=0.493) (Figure 7E and Supplementary Figure 5).
Of note, an increase in genes that regulate the browning of the SAT adipose tissue was also observed after weight loss as well as gene associated with mitochondrial activity (Figure 8A-B and Supplemental Figure S4E-F).

The increase of *CIDEA* gene expression was also assessed by immunofluorescence (Figure 8C and Supplemental Figure S5). The specific staining for CIDEA confirmed the mRNA expression results and showed a strong increase in SAT tissue obtained from a biopsy after weight loss (CIDEA Fluorescence intensity=40.7±2.6 AU) compared to the staining from the baseline biopsy (CIDEA Fluorescence intensity=26.8±2.6 AU; p value baseline vs after weight loss = 0.0098). It should be noted, that all the follow-up biopsies were done during the warm season (spring-summer), which would exclude the possibility that the observed browning/beiging of the white adipose tissue was due to any seasonal effects.

**PLIN1 but not ATGL phosphorylation regulates SAT adipocytes cell size after weight loss.**

Since we found that the post-translational activation of the lipolytic proteins is the key indicator of the lipolytic activity in adipocytes, we investigated the level of pATGL and pPLIN1 in SAT samples obtained at baseline during bariatric surgery and follow-up biopsy (Figure 9A-B). Surprisingly, we observed that after weight loss, there was a slight reduction in the ATGL phosphorylation. On the other hand, this depot showed a strong and significant over-expression of the total form of PLIN1 (that was almost not express in SAT before weight loss) (Figure 9C), and consequently we were able to detect the phosphorylation of PLIN1 after weight loss. These data indicate that during the weight
loss the reduction of adipocyte size and therefore adipocyte lipolytic activity may be mainly under control of PLIN1.
DISCUSSION

The present study provides insights into the potential underlying differences in the cellular and transcriptomic profiles of paired biopsy samples from the omental (omVAT) and superficial abdominal (SAT) depots, from a group of adolescent girls with severe obesity and with a spectrum of insulin sensitivity, undergoing sleeve gastrectomy (SG). Following SG, weight changes were closely monitored in the entire group and in a subset of subjects, a repeated SAT biopsy was performed and measurements of cellularity and gene expression were repeated, during weight stabilization.

The major findings, summarized in Figure 10, are as followed:

First, at baseline, before SG, we found that omVAT compared to SAT displayed: 1) smaller adipocytes and increased lipolytic activity; 2) reduction of pro-inflammatory markers; 3) increased markers of browning/beige of white adipose tissue, and 4) opposite from SAT, omVAT adipocytes peak diameter did not correlate positively with insulin sensitivity.

Second, after SG-induced weight loss, all subjects showed: 1) a marked reduction in body weight, absolute BMI and % change in BMI, reaching after six months a plateau in body weight loss which was maintained throughout the 10-months follow-up visit; 2) a marked improvement in insulin-sensitivity, particularly in the obese girls that were more insulin resistant.

Third, in the 4 subjects who agreed to have a repeated SAT biopsy after weight loss we found: 1) reduction in cells diameter compared to the baseline biopsy and improvement
in insulin-sensitivity; 2) increased in lipolytic activity; 3) strong induction of browning markers; 4) and a significant reduction of pro-inflammatory markers.

A unique aspect of this study is due to the particular young age of the subjects who were studied during adolescence, a developmental and maturational stage during which the adipose tissue experiences not only changes in body fat distribution but also a great accretion in fat mass. Indeed, adolescence is considered a critical time for obesity development (41). Given the major sex differences in body fat distribution (42-45) and higher prevalence rates of severe obesity among females, we elected to study only girls. There is very limited information regarding the differential differences in the cellularity and transcriptomic profiles of the omental and SAT depots from obese adolescents with severe obesity. Likely, due to the greater plasticity of the white adipose tissue at this particular young age (46-48), the findings in the cellularity, the transcriptomic profiles and their responses to SG induced weight loss, may be specific to this developmental stage of adolescence, compared to those described in older subjects with obesity.

**Inter-depot differences in cell size.**

Adipocyte from the omental depot (omVAT) were smaller compared to those from the SAT. These cell size differences are consistent with those reported from adult obese women (49). Surprisingly, we found no relation between cell size and insulin sensitivity in the omVAT in contrast to the significant correlation with the large cell diameter and insulin sensitivity in the SAT. This finding would support the primary role of the SAT rather than
the omVAT in the development of insulin resistance at this particular developmental stage.

Further analysis of the cellular patterns suggests that in adolescents with severe obesity, the proportion of small/large cells and the percentage of small cells from the SAT depot were significantly higher compared to those seen from the omVAT depot (see Table 2). Of note, the cell diameter of the adipocytes from the omVAT depot positively correlated with ratio of small to large cells from the SAT \( (r=0.754, \, p=0.015, \, \text{Figure 1D}) \). The reason for the differential small cell accumulation between the two depots is not clear, but could be related to the regional differences of fat tissue and presence of insulin resistance. To some extent, our results regarding the inter-depot differences in the proportion of small/large cells are different from those reported in adults by Liu et al. Likely, this discrepancy is due to the difference in the age of the subjects studied, that is adolescent vs adults (49).

To the best of our knowledge, our study may be the first to document, using Multisizer technology, that increasing cell size in omVAT is associated with accumulation of small cells in SAT in adolescent girls with severe obesity. Additionally, it also suggests that the cell diameter of the adipocytes from the omVAT depot may regulate the proportion of the small to large cells in the SAT (see Figure 1). Regardless of the directionality of these relations, what is emerging is that the presence of large cells and the greater proportion of small to large cells in the SAT may be playing a greater role in shaping the onset of insulin resistance compared to that seen in the omVAT. Whether this difference is due to the particular maturational stage or due to the regional site of the depot, remains unclear.
To further understand the inter-depot differences in cellularity, we examined whether differences in the functional/activity at the protein level of two of the master regulators in lipolysis, ATGL and HSL, would emerge between the two depots and whether the phosphorylation of these key proteins would be correlated with adipocyte size. To this end, we compared the protein expression level of pATGL and pHSL in SAT and omVAT. In particular, we found a significant increase in pATGL in omVAT compared to SAT (p<0.01), and a trend in increasing HSL phosphorylation which did not reach statistical significance (Figure 5A-C). Surprisingly, in SAT pATGL levels significantly correlated with cell diameter (r=0.742, p=0.042) but not in omVAT (Figure 5D) and notably, both SAT and omVAT pATGL strongly correlated with HOMA-IR (Figure 5E). No significant correlations were observed between pHSL and cells peak diameter or HOMA-IR in both omVAT and SAT (data not shown). Although phosphorylation of the ATGL would suggest a greater lipolytic activity of the omVAT, the fact that pATGL significantly correlated with SAT cell size diameter and HOMA-IR, supports the idea of the SAT as having a primary role in the development of insulin resistance, at least during adolescence.

**Differential transcriptomic profiles between omVAT and SAT depots.**

The use of both RNA-seq and RT-PCR allowed further understanding of the inter-depot differences at the molecular levels by assessing potential differences in key pathways essential to regulation of lipolysis/adipogenesis, browning/beige and inflammation. While no significant differences between the two depots emerged regarding the regulatory genes for lipolysis and adipogenesis, we found differences in the phosphorylation of
pATGL in the omVAT compared to SAT, consistent with a greater lipolytic activity of the omVAT depot (50-52), even at this stage.

Despite the general thinking that the amount of brown adipose tissue shortly declines right after birth, several studies demonstrated the presence of brown-like adipocyte in white adipose tissue also in kids and adults (53-55). Besides BAT, white adipocytes are also reported to be able to “convert” to brown-like cells in response to appropriate stimuli such as different hormones and cold exposure (56, 57). However, the depot-specific expression of browning genes in human white adipose tissue is till poorly investigated. Therefore, of particular interest are the differences in the genes regulating the browning/beige and inflammation in these two depots (see Figure 4). First, we found that the markers of gene regulating the browning of WAT were significantly upregulated in the omVAT compared to SAT (Figure 4A-C).

In accordance with our data, other studies showed in adults that omVAT did exhibit a distinct increase in UCP1 gene expression compared to SAT (58, 59). However, gene expression of other browning/beige markers in human white adipose tissue obtained from adolescents with severe obesity has not yet been described.

Moreover, the higher expression of PGC1a, TBX1, and EBF2 were found to be related inversely and strongly to the omVAT cell diameter. In other words, the larger the cells in omVAT the lower was the level of gene expression (Figure 4). These findings would suggest that there might be a relation between cell size in the omVAT and the activity of browning in this depot. At this point, however, this statement is purely speculative as we have no data on whether the activation of the browning process in omVAT depot was present, as it was not assessed.
In contrast, in the SAT we found no evidence at baseline for the browning/beige markers. As for the genes regulating inflammation, we found that CD68 as well as many of the pro-inflammatory genes were upregulated in the SAT as opposed to the omVAT, these differences were seen both by RNA-seq and confirmed by RT-PCR. Consistent with studies from Leibel et al. in obese adolescents (60), our data showed similar trend but more robust reduction of CD68 and other pro-inflammatory markers in SAT depot. However, these data are in contrast with those reported in adults (49). Several papers reported that in a population of obese adults, visceral adipose tissue exhibit an evident increase of infiltrating macrophages as well as an elevated expression and secretion of pro-inflammatory cytokines (such as IL6, and MCP1) compared to SAT (49, 61-64). These differences in omVAT/SAT inflammation profiles between obese youths and adults is another aspect that strengthen the idea that adolescence and adulthood are two metabolically different stages of human life.

**Effects of SG induced weight loss on cell size and gene expressions from the SAT.**

All subjects lost a substantial amount of fat mass after the first six months following SG. Thereafter, the weight remained stable until the end of the study. As expected, insulin sensitivity also improved significantly in parallel with resolution of prediabetes and type 2 diabetes. These remarkable clinical and metabolic improvements are very similar to those described in the young subjects from the Teen-LABS (18, 65).

The novelty of the current study, relies on the mechanisms causing the observed changes in metabolism. First, we found a profound change in the cell size of the SAT of these girls after weight loss. Second, the lipolytic activity as measured by WB showed a decreased
in ATGL but an increase in PLIN1 phosphorylation. At baseline, PLIN1 was expressed and phosphorylated at very low levels in SAT adipocytes therefore the lipolysis was regulated almost exclusively by ATGL phosphorylation. On the contrary, after weight loss PLIN1 was re-expressed and phosphorylated. It was therefore able to lead lipolysis in combination with ATGL (that almost did not change in phosphorylation level after weight loss) (see Figure 9). The increase in total PLIN1 expression after weight loss that we observed in our cohort was in accordance with previous observations in SAT obtained from adults (64, 66-68).

Third, and perhaps the most novel findings are the induction of the gene markers related to browning/beige of white adipose tissue (see Figure 8) and the down-regulation of the inflammatory genes in the SAT after weight loss (see Figure 7).

The down-regulation of inflammatory genes in SAT after weight loss that we observed in our cohort of adolescent girls are in accordance with the observation of other groups that showed a reduction in inflammatory markers such as CD68 and MCP1 after surgery or after caloric restriction (65, 69-71). In contrast, others described a reduction or even no significant changes in IL6, TNF, or IL18 after weight loss (72, 73).

Similar disagreement was observed in relation to the over-expression of gene markers of the browning of white adipose tissue in SAT induced after weight loss. In fact, while some groups confirmed the presence of beige adipocytes in SAT after weight loss (74), others showed a reduction in key genes associated to browning process (70).

A number of studies in mice have shown that activation of browning/beige of white adipose tissue facilitates weight loss, ameliorates insulin resistance, and corrects hyperlipidemia in obese state (75-78) Other studies in mice have demonstrated that
browning/beiging process was induced by caloric restriction, mediated mainly through IL4 and other M2-polarized macrophages cytokines signaling (79-81). It’s also important to highlight that Zuriaga et al clearly describe an inverse pattern of browning gene expression between mice and humans, suggesting that extrapolation of data from mice on adipose biology to humans should be taken with some caution (82). Consistent with other few clinical studies done in human adults, our findings showed an increase of browning/beige markers in omVAT that led to an improvement of oxidative metabolism and decrease of body fat mass (75, 82, 83). However, in our cohort, the browning activation happens at a much younger age. These opposite changes in the regulatory browning genes and inflammatory genes would suggest modulatory effects on the changes in insulin sensitivity.

Our finding showing the induction of browning/beige in the SAT after SG induced weight loss is in contrast to those reported in adults after caloric restriction (84). A recent study in a large cohort of adult obese individual showed that caloric restriction and diet-induced weight loss diminishes browning features in SAT and that diet-induced changes in body fat are independent of subcutaneous abdominal WAT browning (84). The result and conclusion of the lack of activation of browning in the SAT depot induced diet-induced weight loss cannot be extrapolated to bariatric surgery-induced weight loss. Indeed, several studies recently reported bariatric surgery-induced BAT activation and WAT browning in the neck (85-88), suggesting perhaps that activation of WAT browning may be among one of the many potential mechanisms by which bariatric surgery causes weight loss.
Our study limitations are due to the small number of subjects undergoing SG and consenting to have a repeated follow-up biopsy. Moreover, the limited amount of tissue collected during the biopsies limited our ability to perform further functional assays/experiments to assess browning activity and in vivo/vitro lipolysis assay. We were able to measure only the mRNA expression of the pro-/anti-inflammatory markers in this study, that could potentially be different from protein secretion although in literature it was suggested that mRNA of adiponectin and IL6 correlates well with the protein secretion (89-92). Furthermore, in our study we did not have paired omental and subcutaneous adipose tissue biopsies from a non-caloric restricted group. Also, we did not have a non-surgically treated group (weight loss induced by caloric restriction only), in order to identify if the observed metabolic changes were due to caloric restriction or sleeve gastrectomy.

In conclusion, this study demonstrates that in adolescent girls with severe obesity there are radical differences in the cellular and transcriptomic profiles of paired biopsy samples from the omental (omVAT) and superficial abdominal (SAT) depots. We were able to identify in the omVAT a specific profile consisting in the increase of browning/beige markers and decrease of pro-inflammatory markers. Of note, following weight loss, the SAT depot cellular and transcriptomic profile changed profoundly and was associated with a reversal in insulin resistance.
METHODS

**Study design and subject characteristics.** From “The Yale Study of Body Fat Patterning in Obese Adolescents”, we recruited 10 girls with class 2/3 obesity (7), who elected to have bariatric surgery as weight loss intervention. These 10 obese female patients were between 16 and 22 years old (7 adolescents and 3 young adults), underwent sleeve gastrectomy (SG), and agreed to have a paired subcutaneous periumbilical (SAT) and visceral-omental (omVAT) adipose tissue biopsy on the same day of the surgery procedure at Yale done by our Pediatric Bariatric Surgeon, Dr Geoffrey S. Nadzam. Body weight and metabolic/clinic parameters were monitored during follow-up visits in nine patients, one patient left the study/follow-up after surgery for personal reasons. Four patients consented to have a second SAT biopsy after weight stabilization following the weight loss. The follow-up biopsies were performed at Yale Center for Clinical Investigation-Hospital Research Unit (YCCI-HRU) after administration of local anesthesia (lidocaine without adrenaline/epinephrine).

The clinical characteristics of enrolled subjects are described in Table 1.

**Analytical Methods.** Plasma glucose levels were measured using the YSI 2700 STAT Analyzer (Yellow Springs Instruments), and lipid levels were measured using an autoanalyzer (model 747-200; Roche-Hitachi). Plasma insulin, adiponectin, and leptin levels were measured using radioimmunoassay (Linco, St. Charles, MO).
Adipocyte size measurement. Samples of abdominal subcutaneous and visceral-omental adipose tissue were collected during sleeve-gastric surgery. Two 20–30 mg samples were immediately used for adipose cell size distribution analysis by osmium tetroxide fixation (Multisizer 4; Beckman Coulter, Miami, FL). We performed a curve-fitting analysis technique as previously described (27). We calculated the ‘peak diameter’ of the large adipocytes as defined as the mean diameter that showed the highest frequency of the large cell population. In addition to determining the “peak diameter” of the large adipose cells as described, we calculated the “% of adipose cells above” (% large cells) and “% below” (% small cells) the nadir.

Real time PCR. Fat tissues were homogenized into QIAzol Lysis Reagent (Qiagen). Total RNA was isolated using RNeasy® Mini Kit (Qiagen) and reverse-transcribed to cDNA using High Capacity cDNA RT kit (Applied Biosystems). Real-Time PCR was performed using SYBR green master mix (BioRad) on an Applied Biosystems 7500 Fast Real-Time PCR System. The expression of each gene was normalized to the expression of the housekeeping gene TATA-Binding Protein (TBP). All reactions were performed in triplicate. The relative expression levels of each transcript were calculated using the $2^{-\Delta\Delta Ct}$ method and values were expressed in arbitrary units (AU). The list of all primers used is in Table S1.

RNA next generation sequencing and analysis. The total RNA isolated from SAT and omVAT depots was measured using an Agilent 2200 Bioanalyzer (Agilent) to evaluate
quality and quantity. RNAs with RIN>8.0 were used to construct the cDNA library and sequencing was subsequently performed with Illumina Hi-Seq 4000 (Illumina). The sequencing reads for each of the samples were aligned to the GRCh38 human reference using HISAT2 (93). Then, gene-level read counts were generated using the featureCounts function of Rsubread (94), based on annotations from the ENCODE v27 GTF file. Differential gene expression was performed using DESeq2 (95). The DESeq2 analysis results were submitted to the Ingenuity Pathway Analysis software (IPA, QIAGEN), and a Core Analysis was used to perform pathway enrichment analysis on the differentially expressed genes. RNA-Seq data will be deposited into GEO, accession GSE145284.

**Western Blotting.** Protein extraction from fat tissues was performed using RIPA buffer supplemented with phosphatase (PhosSTOP, Roche) and proteinase (cOmplete MINI, Roche) inhibitors and protein content was quantify using BCA assay (Pierce). After denaturation with heat and beta-mercaptoethanol, an equal amount of proteins was run in 4-12% Tris-Glycine Gel (Novex, Invitrogen). Proteins were transferred into PVDF membrane (Millipore) by semi-dry transfer and blocked with 3% BSA. Membranes were blotted overnight at 4°C with specific primary antibody. Actin (#4967), ATGL (#2138), HSL (#4107), pSer660 HSL (#4126), Perilipin-1 (#3467) were from Cell Signaling, pSer406 ATGL (Ab-135093) was from Abcam, and pSer522 Perilipin-1 (#4856) from VALA Sciences. Membranes were washed in TBS-T three times and then incubated 1 hour with HRP-conjugated secondary antibody (Cell Signaling). After three washing in TBS-T, the specific band was visualized using enhanced ECL chemiluminescence (Pierce). Film
were developed within the linear dynamic range of signal intensity and then scanned. The intensity of the bands was measured using ImageJ software.

Because of limited amount of SAT tissue collected, the protein expression was performed on $n = 8$ SAT tissue collected during the sleeve gastrectomy.

**Immunofluorescence and Immunohistochemistry (IHC).** Adipose tissue biopsy samples were used for immunofluorescence staining for browning markers (UCP-1 R&D Systems MAB6158; CIDEA-FITC Bioss bs-7649R). Formalin-fixed and paraffin-embedded tissue blocks were deparaffinized and rehydrated. Section were rinsed in PBS before epitope retrieval with either 10mM citrate buffer (pH 6.0). After blocking in 5% BSA at room temperature, sections were then incubated overnight at 4C with primary antibodies. After rinsing in PBS-tween, sections were incubated with specific fluorescent secondary antibodies Alexa-Fluo-488 (Invitrogen) and mounted with Prolong Antifade mounting medium with DAPI (Invitrogen). Sections stained with secondary antibody while omitting the primary antibody served as negative controls. Images were acquired using Leica SP5 confocal microscope (Leica, Germany) using the following settings: Blue fluorescence: 405 Laser power = 16%, gain = 1041, Green fluorescence: 488 Laser power = 20%, gain = 30; all images were acquired using the same pixel dwell time settings = 721 nanoseconds.

Immunohistochemistry staining was performed using a standard protocol on sections from formalin-fixed, paraffin-embedded tissue blocks. Briefly, sections were deparaffinized, rehydrated, and treated with 10 mmol/L citrate buffer (pH 6.0) in a steamer, and then endogenous peroxidase was blocked with 3%H2O2. The sections
were then incubated for 1 h at room temperature with primary antibodies, mouse monoclonal anti-CD68 (Ab-3 clone KP1; Thermo Fisher Scientific). After rinsing in Tris-buffered saline solution containing 0.25% Triton X-100 (pH 7.2), sections were incubated with ENVISION+ (K4007 or K4011; DAKO, Carpinteria, CA), followed by visualization with 3.39-diaminobenzidine tetrachloride (DAKO). All sections were counterstained with GILL III hematoxylin, dehydrated, and coverslipped with a resinous mounting media. Images were acquired using Aperio digital whole slide scanner and representative images were presented.

**Statistical analysis.** Before analysis, the data were tested for normality. When appropriate, log transformed data were tested with 2-tailed Student’s t-test. Weighted means for adipocytes were calculated for adipose cell size of the abdominal and visceral fat. Wilcoxon matched-pairs signed rank test was applied, otherwise specified. Correlation tests were performed by linear regression test and by non-parametric Spearman correlation analysis. For all analysis a p value<0.05 was considered statistically significant. Data are expressed as means and SEM. GraphPad Prism 8.2 (San Diego, CA) was used for all statistical analysis.

**Study Approvals.** The nature and potential risks of the study were explained to all subjects before obtaining their written informed consent. The study was approved by the ethics committees of the Yale University Hospital (HIC1109009034 and HIC1503015459) and registered on ClinicalTrials.gov (NCT02004561 and NTC02395003, respectively).
AUTHOR CONTRIBUTIONS

E.T. performed all experiments, data analyses and wrote the manuscript. J.N. processed the tissues and performed cell size measurements. M.V.N. and R.G. recruited the subjects. B.P. obtained informed consent. G.S.N. performed the gastric sleeve surgery and provided the fat tissue biopsies. A.V.-M. collected the follow-up biopsies. J.K. performed the RNA-seq analysis and help for the interpretation of the analysis. G.I.S. provided laboratory space and help with the setup of the used methods. S.C. designed the study and wrote the manuscript. All authors contributed to the interpretation of the data. S.C. is the guarantor of this work, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
AKNOLEGMENTS

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REFERENCES


Figure 1. Multisizer adipose cell profile from subcutaneous adipose tissue (SAT) and omental depot (omVAT). (A) Multisizer cell profiles for SAT and omVAT depot collected from 10 girls with severe obesity undergoing sleeve gastrectomy using the mean parameters from the curve-fitting formula, and adipocyte peak diameter (in insert). (B) Correlation between HOMA-IR and SAT or omVAT cell peak diameter (n=9). (C) Strong correlation between omVAT adipocytes peak diameter and percentage of small cells in SAT (n=10). (D) Strong direct correlation between omVAT adipocytes peak diameter and ratio small to large cells in SAT (n=10).
**Figure 2. Transcriptome analysis in SAT and omVAT.** (A) RNA sequencing (RNA-seq) analysis of SAT and omVAT with heatmap and clustering of 4155 most variably expressed genes in the RNA-seq dataset. (B) PCA analysis of the significant genes. (C) Canonical pathways enrichment analysis for up-regulated (in yellow) and down-regulated (in blue) genes between SAT vs omVAT samples. The bar represents the percent of gene up-/down-regulated within the specific canonical pathways. The solid line represents the -Log(p value) for each pathway. Analysis of n=10 samples each depot.
Figure 3. Evaluation by real-time PCR of a panel of genes associated with lipid metabolism, adipogenesis, browning of white adipose tissue, and inflammation in SAT and omVAT. Validation by real time PCR of RNA-seq results of a panel of genes involved in (A) lipid metabolism, (B-C) adipogenesis, and (D) inflammation (n=10). (E) Representative images of CD68 macrophage immunohistochemistry staining confirming a slight reduction in macrophage infiltration in omVAT compared to SAT. Scale bar: 200 µm.
Figure 4. Distinct profile for browning markers in omVAT compared to SAT depots. Validation by real time PCR of RNA-seq results of a panel of genes involved in (A) browning of white adipose tissue. Strong inverse correlation between omVAT adipocyte peak diameter and (B-D) browning gene expression or (E-G) mitochondrial markers (n=10). (H) Representative immunofluorescent staining images showing an increase of UCP1 in omVAT adipose tissue compare to SAT. Scale bar: 50\(\mu\)m.
Figure 5. Increase in lipolytic activity in omVAT mainly through ATGL phosphorylation. (A) Representative blots showing ATGL and HSL phosphorylation in SAT (n=8) and omVAT (n=10). (B-C) Quantification of the phosphorylated/total ratio both of ATGL and HSL. (D) Positive correlation of ATGL phosphorylation with adipocytes peak diameter in SAT but not omVAT. (E) Positive correlation of ATGL phosphorylation with HOMA-IR in SAT (n=8) and omVAT (n=9). (F) Correlation of ATGL phosphorylation with percentage of small cells adipocytes peak diameter in SAT (n=8) but not omVAT (n=10).
Figure 6. Reduction in body weight, improvement in insulin sensitivity and changes in adipocyte diameter after weight loss. (A) Reduction in body weight as percent change in BMI from baseline (dotted line). Each solid line represents an individual participant (n=9) during the follow-up visits. (B) Improvement in insulin sensitivity after weight loss (n=6). (C) Multisizer cell profiles in SAT as mean of 4 subjects at the surgery time and returning for a second SAT biopsy after weight loss. (D) Adipocyte peak diameter from SAT at baseline biopsy and at the follow-up biopsy after weight loss (n=4).
Figure 7. Variation in the expression of genes associated to lipid metabolism, adipogenesis, and inflammation in SAT after weight loss. Validation by real time PCR of RNA-seq results of a panel of genes involved in (A) lipolysis, (B-C) adipogenesis, and (D) inflammation (n=4). (E) Representative images of CD68 macrophage immunohistochemistry staining confirming the mRNA expression levels in SAT at baseline and after weight loss. Scale bar: 200\(\mu\)m.
Figure 8. Upregulation of browning genes in SAT after weight loss. Validation by real time PCR of RNA-seq results of a panel of genes involved in (A) browning of white adipose tissue and (B) mitochondrial markers (n=4). (C) Representative immunofluorescent staining shows an increase of CIDEA in SAT adipose tissue after weight loss (follow-up biopsy) compare to the baseline biopsy. Scale bar: 50μm.
Figure 9. Switch of lipolytic activity towards activation of PLIN1 phosphorylation in SAT after weight loss. (A) Protein expression of both total and phosphorylated ATGL and PLIN-1 in SAT before (Baseline) and after weight loss (Follow-up) (n=4). (B) Quantification of ATGL phosphorylated/total ratio at baseline and follow-up biopsy (n=4). (C) Quantification of PLIN1 total/actin ratio at baseline and follow-up biopsy (n=4).
Figure 10. Summary of the key study findings at Baseline before the bariatric surgery and after weight loss.
| TABLES |
|-------------------|-------------------|
| **Tables**        |                   |
| **Mean**          | **Range**         |
| **Age (Years)**   | 18.3              | 16-22 |
| **Ethnicity (nH/H)** | 4/6               |
| **Tanner stage (IV/V)** | 5/5               |
| **Anthropometrics** |                  |
| **Weight (Kg)**   | 125.7             | 96.9-178.7 |
| **Height (cm)**   | 165.1             | 155-185.4 |
| **BMI (Kg/m²)**   | 45.9              | 36.7-57.2 |
| **% Fat Mass**    | 48.8              | 42.8-56.6 |
| **Class of Obesity (2/3)** | 1/9               |
| **Metabolic measurements** |            |
| **Fasting glucose (mg/dL)** | 87.3             | 69-100  |
| **2hr glucose (mg/dL)** | 130.4            | 80-202  |
| **Fasting insulin (µU/mL)** | 33.13            | 9.4-71  |
| **HOMA-IR**       | 6.99              | 0.5-17.51 |
| **Leptin (ng/mL)** | 127.21            | 84.38-223.66 |
| **Lipids**        |                   |
| **Total Cholesterol (mg/dL)** | 167.9           | 121-261 |
| **HDL (mg/dL)**   | 45                | 27-57   |
| **LDL (mg/dL)**   | 101.6             | 61-190  |
| **TG (mg/dL)**    | 113.4             | 40-256  |
| **FFA (µmol/L)**  | 0.574             | 0.338-0.765 |
| **Liver**         |                   |
| **ALT (U/L)**     | 32.3              | 14-96   |
| **AST (U/L)**     | 23.5              | 17-42   |

**Table 1. Clinical and metabolic characteristics of the obese adolescents undergoing bariatric surgery.** Demographic, anthropometric and metabolic characteristics are shown for 10 obese adolescent females undergoing sleeve-bariatric surgery. nH: non Hispanics; FFA: free fatty acid; H: Hispanics; TG: triglycerides.
Table 2. Comparison of adipose cell size variables in SAT and omVAT depot. Data are expressed as mean ± SEM. The non-parametric 2-tailed paired Student’s t-test was used for comparing SAT vs omVAT and p values<0.05 were considered significant (in bold).

<table>
<thead>
<tr>
<th>Cell size variables</th>
<th>SAT</th>
<th>omVAT</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak diameter (μm)</td>
<td>129.73±4.12</td>
<td>104.60±5.85</td>
<td>0.002</td>
</tr>
<tr>
<td>Fraction of large cells</td>
<td>0.68±0.07</td>
<td>0.96±0.10</td>
<td>0.002</td>
</tr>
<tr>
<td>Nadir (μm)</td>
<td>79.61±3.35</td>
<td>61.06±4.04</td>
<td>0.002</td>
</tr>
<tr>
<td>Ratio small/large cells</td>
<td>0.90±0.11</td>
<td>0.56±0.10</td>
<td>0.006</td>
</tr>
<tr>
<td>Percentage of small cells</td>
<td>45.23±3.64</td>
<td>33.38±4.45</td>
<td>0.01</td>
</tr>
<tr>
<td>Median (μm)</td>
<td>85.19±4.69</td>
<td>83.13±3.67</td>
<td>0.84</td>
</tr>
<tr>
<td>Mean (μm)</td>
<td>88.21±2.47</td>
<td>83.40±2.90</td>
<td>0.23</td>
</tr>
</tbody>
</table>
Table 3. Comparison of anthropometric characteristics between all follow-up and selected returning patients. All values are expressed as mean ± SEM. *p value of the returning 4 girls compared with all girls at the same follow-up time. BW, body weight; HT, height; % FAT, percentage of fat.