Cellularity and Adipogenic Profile of the Abdominal Subcutaneous Adipose Tissue From Obese Adolescents: Association With Insulin Resistance and Hepatic Steatosis

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OBJECTIVE—We explored whether the distribution of adipose cell size, the estimated total number of adipose cells, and the expression of adipogenic genes in subcutaneous adipose tissue are linked to the phenotype of high visceral and low subcutaneous fat depots in obese adolescents.

RESEARCH DESIGN AND METHODS—A total of 38 adolescents with similar degrees of obesity agreed to have a subcutaneous periumbilical adipose tissue biopsy, in addition to metabolic (oral glucose tolerance test and hyperinsulinemic euglycemic clamp) and imaging studies (MRI, DEXA, ^{1}H-NMR). Subcutaneous periumbilical adipose cell-size distribution and the estimated total number of subcutaneous adipose cells were obtained from tissue biopsy samples fixed in osmium tetroxide and analyzed by Beckman Coulter Multisizer. The adipogenic capacity was measured by Affymetrix GeneChip and quantitative RT-PCR.

RESULTS—Subjects were divided into two groups: high versus low ratio of visceral to visceral + subcutaneous fat (VAT/[VAT+SAT]). The cell-size distribution curves were significantly different between the high and low VAT/(VAT+SAT) groups, even after adjusting for age, sex, and ethnicity (MANOVA $P = 0.035$). Surprisingly, the fraction of large adipocytes was significantly lower ($P < 0.01$) in the group with high VAT/(VAT+SAT), along with the estimated total number of large adipose cells ($P < 0.05$), while the mean diameter was increased ($P < 0.01$). From the microarray analyses emerged a lower expression of lipogenesis/adipogenesis markers (sterol regulatory element binding protein-1, acetyl-CoA carboxylase, fatty acid synthase) in the group with high VAT/(VAT+SAT), which was confirmed by RT-PCR.

CONCLUSIONS—A reduced lipo-/adipogenic capacity, fraction, and estimated number of large subcutaneous adipocytes may contribute to the abnormal distribution of abdominal fat and hepatic steatosis, as well as to insulin resistance in obese adolescents. ***Diabetes*** **59:2288–2296, 2010**

White adipose tissue (WAT) plays a critical role in obesity-related metabolic dysfunctions. Danforth (1) and Shulman (2) raised the hypothesis that inadequate subcutaneous fat stores result in lipid overflow into visceral fat and other nonadipose tissues, which was elegantly explored by Ravussin and Smith (3). Sethi and Vidal-Puig proposed that impaired subcutaneous WAT expandability might cause obesity-associated insulin resistance (4). In adults, increased fat cell size, a marker of impaired adipogenesis, was reported to be related to insulin resistance and predicts the development of type 2 diabetes (5). Recent studies by McLaughlin et al. (6) reported in adults that an increase in the proportion of small adipocytes, but not increased fat cell size, and an impaired expression of markers for adipogenesis are related to insulin resistance. Little is known about adipocyte size and adipogenic capacity during adolescence, a time when the expansion of WAT results from combined adipocyte hypertrophy and hyperplasia. In contrast, adult adipocytes exhibit a remarkably constant turnover (7). Recently, we described a group of obese adolescents presenting with a reduced subcutaneous abdominal fat depot, increased visceral fat, hepatic steatosis, and marked insulin resistance (8). Building on these findings, we asked the following question: is the adipogenic capacity of the abdominal subcutaneous fat depot in obese adolescents associated with a decreased proportion of large adipose cells and reduced expression of genes regulating adipocyte differentiation? We hypothesized that, in some obese adolescents, the lack of expandability of the subcutaneous abdominal fat might be linked to adipocyte size, its adipogenic expression, and the fat accumulation in liver and muscle. To test this hypothesis, we used metabolic and imaging techniques, together with direct measurements of adipocyte size and gene expression, in two groups of obese adolescents with marked differences in the proportion of visceral to subcutaneous abdominal fat.

RESEARCH DESIGN AND METHODS

The Yale Pathophysiology of Type 2 Diabetes in Obese Youth Study is a long-term project aimed at examining early alterations in glucose metabolism in relation to fat patterning in obese adolescents. As part of this study, all subjects undergo a detailed assessment of abdominal fat distribution by

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Received 25 January 2010 and accepted 5 May 2010.

This publication was and its contents are solely the responsibility of the authors and do not necessarily represent the official views of National Center for Research Resources or National Institutes of Health.

DOI: 10.2337/db10-0113

See accompanying commentary, p. 2105.

TABLE 1
Clinical characteristics of the obese adolescents (N = 38)

	Low VAT/(VAT + SAT) ratio (0.08 ± 0.01)	High VAT/(VAT + SAT) ratio (0.15 ± 0.04)	P value	Adjusted P value (age, race, sex)
n	20	18		
Age (years)	15.5 ± 2.8	14.7 ± 2.4	0.357	
Sex (female/male)	14/6	10/8	0.357#	
Race (C/AA/H)	3/10/7	8/4/6	0.089#	
Tanner stage (II-III/IV-V)	6/14	7/11	0.873#	
Anthropometrics				
BMI (kg/m^2)	37.0 ± 8.1	36.6 ± 6.8	0.851	0.636
% fat	40.7 ± 3.6	38.6 ± 6.0	0.262	0.322
Lean body mass (kg)	56.9 ± 11.5	55.4 ± 9.9	0.717	0.593
Waist (cm)	105.0 ± 19.0	108.1 ± 13.5	0.585	0.732
Systolic BP	115.4 ± 10.9	119.9 ± 10.6	0.228	0.235
Diastolic BP	66.6 ± 8.6	71.8 ± 8.8	0.093	**0.031**
Body fat distribution				
Abdominal				
Visceral fat (cm^2)	55.0 ± 26.3	91.7 ± 31.1	**0.000**	**0.007***
Subcutaneous fat (cm^2)	603.7 ± 230.8	520.7 ± 181.5	0.231	0.105*
VAT/(VAT + SAT) ratio	0.08 ± 0.01	0.15 ± 0.04	**0.000**	**0.000***
Ectopic fat content				
IMCL	1.3 ± 0.6	1.2 ± 0.4	0.549	0.454*
HFF (%)	2.7 ± 6.8	10.0 ± 12.0	**0.033**	**0.032***
Metabolic measurements				
Fasting glucose (mg/dl)	96.6 ± 9.0	97.1 ± 7.9	0.860	0.888*
2-h glucose (mg/dl)	115.4 ± 18.7	139.6 ± 34.0	**0.013**	**0.002***
AUC-glucose (mg/dl/min)	116.6 ± 16.8	130.7 ± 27.5	0.086	0.091*
Fasting insulin (μU/ml)	29.0 ± 13.5	39.8 ± 24.2	0.108	0.218*
M (kg/LBM · min)	11.6 ± 5.6	8.1 ± 4.8	**0.046**	**0.043***
Matsuda index (WBISI)	2.5 ± 2.1	1.5 ± 0.9	0.081	0.082*
Adiponectin (μg/ml)	9.3 ± 5.0	6.5 ± 3.9	0.079	**0.037***
Leptin (ng/ml)	31.2 ± 15.1	34.9 ± 15.7	0.489	0.652*
Lipids				
HDL (mg/dl)	43.8 ± 9.2	37.0 ± 7.6	**0.024**	0.163*
TG (mg/dl)	101.2 ± 60.8	136.3 ± 81.7	0.158	0.843*
FFA (μM/l)	501.1 ± 152.8	615.6 ± 180.7	0.072	0.632*

Data are means ± SD. #χ^2; *log transformed; P values <0.05, shown in bold, are statistically significant.

magnetic resonance imaging (MRI). As previously described, we found that the metabolic profile worsens with the increasing visceral to visceral + subcutaneous fat (VAT/[VAT+SAT]) ratio (8). On the basis of the distribution of the VAT/(VAT+SAT) ratio obtained in our entire multiethnic cohort of 141 adolescents (8), we used the 50th percentile (0.11) as a cutoff value to recruit and enroll subjects in the current biopsy study. Thirty-eight obese adolescents agreed to have a subcutaneous periumbilical adipose tissue biopsy and were divided into two groups: low (<0.11) and high (>0.11) VAT/(VAT+SAT) ratio. Their clinical characteristics are described in Table 1. None of the subjects were on any medications nor had any known disease. 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.

Metabolic studies. All subjects were invited to the Yale Center for Clinical Investigation (YCCI) for an oral glucose tolerance test at 8:00 A.M. after an overnight fast. Baseline fasting blood samples were obtained with the use of an indwelling venous line for measurement of glucose, insulin, lipid profile, free fatty acids, adiponectin, and leptin levels. An oral glucose tolerance test was then performed and blood samples were obtained every 30 min for 180 min for the measurement of plasma glucose and insulin as previously described (9). The composite whole-body insulin sensitivity index (WBISI) was calculated using the formula described by Matsuda and DeFronzo (10).

The hyperinsulinemic euglycemic clamp. In the morning at 8:00 A.M., after an overnight fast of 10–12 h, insulin sensitivity was measured by the hyperinsulinemic euglycemic clamp, during which time insulin was administered as a prime continuous infusion of 80 mU/m^2-min for 120 min (11).

Abdominal fat distribution. Whole-body composition was measured by dual-energy X-ray absorptiometry (DEXA) with a Hologic scanner (Boston, MA). Abdominal MRI studies were performed on a GE or Siemens Sonata 1.5 Tesla system (12). Five slices, obtained at the level of the L4/L5 disc space, were analyzed for each subject, as previously reported (13).

Liver fat assessment: fast MRI. Hepatic fat fraction (HFF), an estimate of the percentage of fat in the liver, was measured by fast-gradient MRI (12). HFF was measured in a single slice of the liver; we validated this method against proton magnetic resonance spectroscopy (^{1}H-MRS) and found a strong correlation (r = 0.93, P < 0.001) (14).

Muscle lipid partitioning: ^{1}H-NMR. Localized ^{1}H-NMR spectra of the soleus muscle were acquired on a 4T Biospec system (Bruker Instruments, Billerica, MA, as previously described) (15). Intramyocellular lipid was measured in 20 of the 38 subjects.

Adipocyte size and number. Samples (2 g) of subcutaneous adipose tissue were obtained inferior to the umbilicus after administration of 0.25 lidocaine with adrenaline (epinephrine) for local anesthesia, from which two 20–30-mg samples were used immediately for adipose cell-size distribution analysis by the osmium fixation, Beckman Coulter (Miami, FL) Multisizer III, curve-fitting analysis technique previously described (6). In addition to determining the fraction of large adipose cells (fraclarge) and the "peak diameter" of the large adipose cells as described, the "% of adipose cells above" (% large cells) and "% below" (% small cells) the nadir were calculated.

A secondary end point, the number of subcutaneous adipose cells, was estimated by the following formula: cell number = volume of subcutaneous abdominal adipose tissue/weighted volume per cell. Volume of adipose tissue was obtained from MRI scans, and average volume per cell was calculated as the weighted volume based on the relative number of cells per volume bin in the cell-volume histogram generated by the Multisizer software. We used the following formula: average volume per cell = $\sum {}^4/_3\pi({}^{d_i}/_2)^3 p_i$ (that is, the sum of the volumes corresponding to each bin times the relative frequency (p) of that bin (i) (16). The number of large cells was then calculated by applying the percentage of large cells to the total number of cells.

GeneChip. RNA expression was studied in 34 subjects (16 low and 18 high VAT/[VAT+SAT] group) using Affymetrix Human Gene 1.0 ST arrays, covering 28,869 genes. Total RNA was isolated using TRIzol reagent and was further

purified using an RNeasy kit (Qiagen, Valencia, CA). The quality of total RNA was evaluated by the A260/A280 ratio and by electrophoresis on an Agilent Bioanalyzer. Afterward total RNA was submitted to the Keck Microarray Resource for sample processing and chip hybridization according to the manufacturer's instructions. Data processing is described in the supplementary material available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0113/DC1.

Quantitative real-time PCR. The quantification of several differentially expressed genes by real-time RT-PCR was performed using an ABI 7,000 Sequence Detection system (Applied Biosystems, Foster City, CA). The nucleotide sequences of the primers and PCR conditions are added in the supplementary material available in an online appendix. For each run, samples were run in duplicates for both the gene of interest and 18S. Quantitative analysis was determined by $\Delta/\Delta CT$ method normalized to both a control and 18S message.

In vitro lipogenesis test. Adipose tissue (50 mg) was incubated in 0.5 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5% bovine serum albumin and 2 mmol/l glucose, at 37°C for 1 h in the presence of 0.5 mCi (18.5 kBq) of [U-14C] glucose (Amersham Biosciences) with or without 10 nmol/l insulin. After transferring the fat sample into 1 ml PBS, we stopped the reaction by adding 4 ml of liquid scintillation fluid (Betafluor, National Diagnostics USA, Atlanta, GA). Test tube was incubated overnight at room temperature with vigorous shaking. A 3 ml aliquot of the upper phase was transferred to a scintillation vial, and the radioactivity was measured using a Packard liquid scintillation counter. Lipogenic activity was expressed as nmol of glucose incorporated per h per mg of adipose tissue. The difference between basal and stimulated condition was calculated and expressed as percent increase in lipogenesis.

Analytical methods. Plasma glucose levels were measured using the YSI 2,700 STAT Analyzer (Yellow Springs Instruments, Yellow Springs, OH), and lipid levels were measured using an Autoanalyzer (model 747–200, Roche-Hitachi). Plasma insulin, adiponectin, and leptin were measured with a radioimmunoassay (Linco, St. Charles, MO).

Statistical analysis. Student's unpaired t tests or the χ^2 test (for categorical variables) were used to compare the clinical and laboratory characteristics between the two groups. Potential confounders (sex, age, and race) were entered in an ANOVA model in which the metabolic and cell-size parameters were dependent variables and the VAT/(VAT+SAT) ratio group was the primary grouping variable; nonnormally distributed parameters were log-transformed before entry into the model. The composite difference between the curve profiles of the two groups was assessed via MANCOVA, in which the seven cell-size parameters were dependent variables and the VAT/(VAT+SAT) ratio group was the primary grouping variable, while race and sex were entered as fixed factors and age and BMI were entered as covariates. Comparisons of relative gene expression as quantitated by RT-PCR were compared with Mann-Whitney test. These analyses were performed using SPSS 16 (SPSS, Chicago, IL). For all analyses, a P value <0.05 was considered statistically significant.

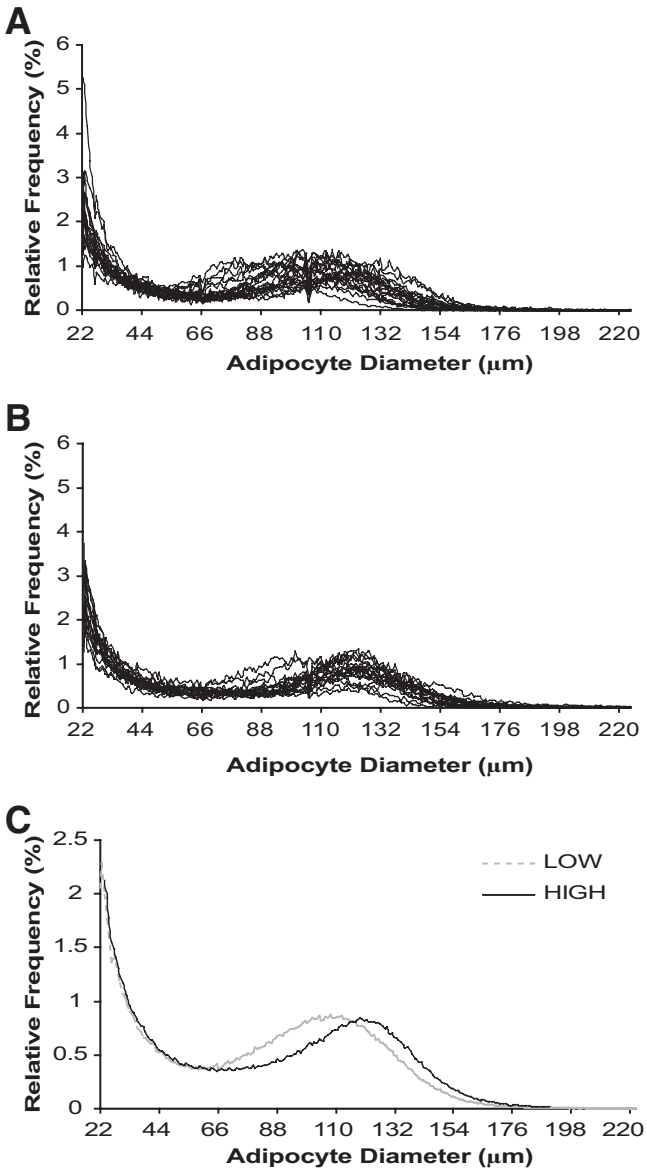

FIG. 1. Multisizer adipose cell profiles of 20 subjects with a low VAT/(VAT+SAT) ratio (*A*) and 18 subjects with a high VAT/(VAT+SAT) ratio (*B*), plotting cell diameter using linear bins against relative frequency in percent *C*: Cell-size profiles of the adipose cell size using the mean parameters from the curve-fitting formula for subjects with a low VAT/(VAT+SAT) ratio (dashed line) and subjects with a high VAT/(VAT+SAT) ratio (solid line) (P = 0.035 using MANCOVA).

RESULTS

Body fat patterning and metabolic characteristics. While both groups were similar in age, sex, % total body fat, and BMI (Table 1), the high VAT/(VAT+SAT) group had a lower percentage of African Americans. Despite similar degrees of overall adiposity, significantly greater visceral and HFF were observed in subjects with a high VAT/(VAT+SAT). Two-h glucose levels were higher in the group with high VAT/(VAT+SAT), who also were found to have lower plasma adiponectin levels and were more insulin resistant (by M/lean body mass) than the low ratio group, even after adjusting for age, race, and sex. Significantly lower HDL levels (P = 0.024) and a trend toward increased fasting free fatty acids (P = 0.072) were found in the group with the high VAT/(VAT+SAT).

Adipose cell-size distribution and number. Fig. 1*A* and *B* illustrates the adipose cell-size distribution profile for each individual in the low and high ratio groups, and Fig. 1*C* shows representative adipose cell-size profiles for the two groups using the average parameters determined by the curve-fitting analyses. The two curves are distinctly different, showing a shift to the right in those with a high ratio. MANOVA analysis applied to the seven curve-fitting parameters showed that the overall cell-size distribution was statistically significantly different between the low and high VAT/(VAT+SAT) groups (P = 0.027). After adjusting for age, BMI, sex, and ethnicity, MANCOVA analysis continued to show a statistically significant difference (P = 0.035).

As shown in Fig. 2, the nadir was significantly shifted (P = 0.001) to a larger peak diameter (P = 0.006), and the fraction of large cells was reduced (P = 0.008) in the high compared with the low group. Figure 2*D* shows the much smaller estimated number of large cells in the subcutaneous fat depot of the high VAT/(VAT+SAT) group (P = 0.044) despite no significant difference in the total numbers of subcutaneous cells (low, $1.4 \times 10^9 \pm 9.3 \times 10^8$; high, $1.0 \times 10^9 \pm 5.3 \times 10^8$; P = 0.144). Using the Spearman correlation, we found that the peak diameter

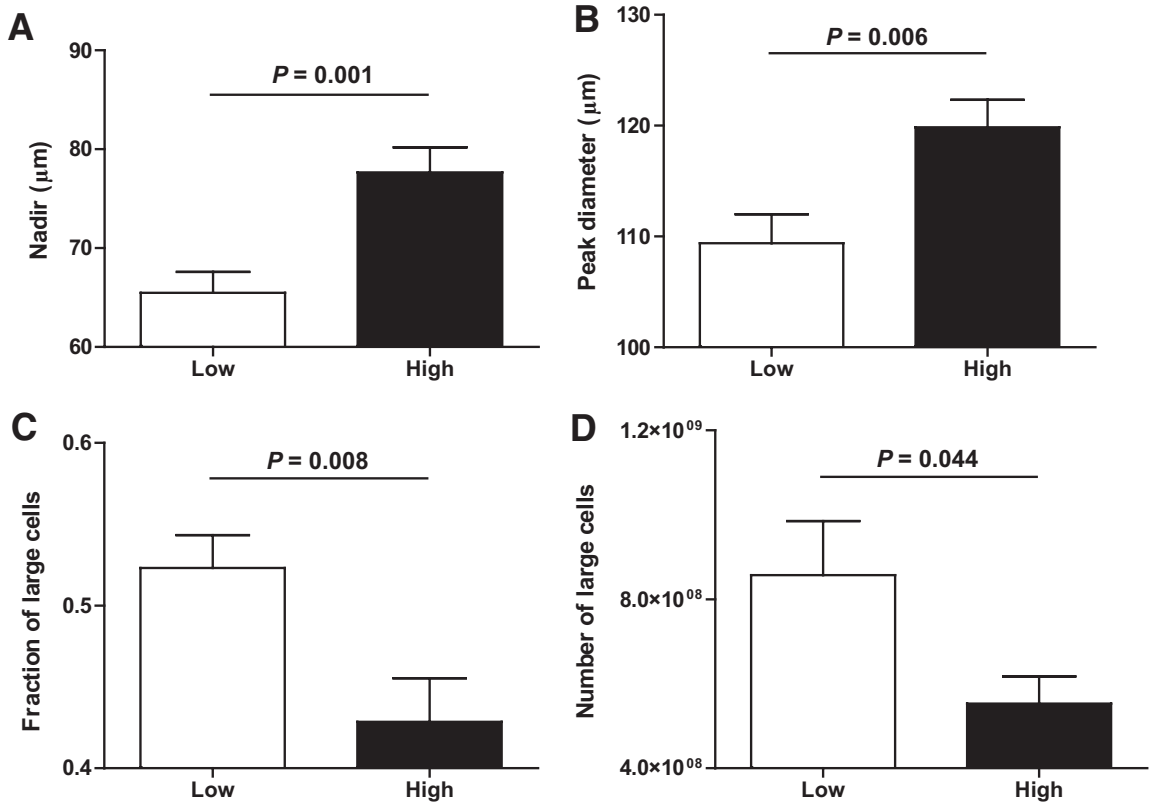

FIG. 2. Differences in cell-size parameters between the groups with the low (white bar) and the high (black bar) VAT/(VAT+SAT) ratio. (*A*) nadir, (*B*) peak diameter (cp), (*C*) fraction of large cells (fraclarge), (*D*) number of large cells (means ± SD).

($r = 0.383$, $P = 0.019$), the fraction of large cells ($r = -0.327$, $P = 0.048$), the nadir ($r = 0.403$, $P = 0.013$), and the number of large cells ($r = -0.398$, $P = 0.015$) significantly correlated with the VAT/(VAT+SAT) ratio, which shows that the correlations previously shown in Fig. 2 are not artifacts of dichotomization into high and low groups.

Gene expression profiles reveal significant changes in lipogenesis genes. Affymetrix Gene Arrays ST 1.0 were used to evaluate potential differences in the gene expression profiles of the subcutaneous fat biopsy specimens. Because ethnicity of the subjects and array batch contributed considerable variation in the ANCOVA model, the data were adjusted for these covariates. Subsequently, the expression data matrix of 28,882 probe sets from 34 GeneChips was subjected to principal component analysis to view the overall trends of the dataset. Principal component analysis showed a clear separation of the high and low VAT/(VAT+SAT) groups (supplementary Fig. 1, available in an online appendix).

To address the question of which pathways contribute to these differences in the gene expression profiles of the high and low VAT/(VAT+SAT) groups, we performed a multivariate analysis of 61 predefined gene sets. Interestingly, the insulin signaling pathway was ranked in position six of the 61 gene sets (Table 2, $P < 0.001$). Of the 142 genes representing this pathway, 74 were characterized by differential expression between the high and low VAT/(VAT+SAT) groups ($P < 0.05$). PathVisio was used for visualization of gene sets detected by the Westfall-Young statistic. Expression differences in genes of the insulin signaling pathway, between the high and low VAT/(VAT+SAT) group, are mapped in Fig. 3. Green indicates the significantly decreased expression observed in the high VAT/(VAT + SAT) group of phosphoinositide 3-kinase (PIK3R3), phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CD), phosphatidylinositol 3-kinase (PIK3R2), protein kinase C, sterol regulatory element-binding protein 1 (SREBF1), fatty acid synthase (FASN), and acetyl-CoA carboxylase (ACACA). In contrast, the expression levels of the insulin receptor, phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CB), fructose bisphosphatase (FBP1), phosphoenolpyruvate carboxykinase 2, and 5′-AMP-activated protein kinase (PRKAA1 and PRKAG1) are characterized by a significantly increased expression in subcutaneous adipose tissue of the high VAT/(VAT+SAT) group in comparison to the low VAT/(VAT+SAT) group (Fig. 3, in red). In the insulin signaling pathway, the highest fold-changes were observed for the following genes: PIK3R3, FBP1, SREBF1, FASN, and ACACA.

Real-time RT-PCR. The Gene Array data of sterol regulatory element binding protein-1 (SREBF1), FASN, and ACACA, as well as four additional insulin signaling–related genes [lipoprotein lipase (LPL), peroxisome proliferator-activated receptor (PPARγ2), lipin 1 (LPIN1), and adiponectin (ADIPOQ)] were further analyzed in adipose tissue of the low and high VAT/(VAT+SAT) groups using real time RT-PCR. The comparisons of the expression data ($2^{\Delta\Delta Ct}$-values) are shown in Fig. 4. Subjects with a high VAT/(VAT+SAT) ratio had significantly lower gene expression of SREBF1 ($P = 0.0057$), FASN ($P = 0.0185$), ACACA ($P = 0.0125$), LPIN1 ($P = 0.0026$), LPL ($P = 0.0069$), PPARγ2 ($P = 0.0025$), and ADIPOQ ($P = 0.0159$) than those with a low VAT/(VAT+SAT) ratio. Using the Spearman correlation, significant negative relationships were found between VAT/(VAT+SAT) ratio and the mRNA

TABLE 2
Top 20 most differential regulated pathways out of 61

Pathway name	*t* value	Westfall-Young adjusted *P* value	No. of genes in pathway
GPCRDB_Class_A_Rhodopsin_like	6.203137e-51	0.001	174
Smooth_muscle_contraction	1.882423e-48	0.001	140
Calcium_regulation_in_cardiac_cells	2.124932e-47	0.001	135
Cell_cycle_KEGG	2.704092e-47	0.001	87
Peptide_GPCRs	3.017272e-47	0.001	72
Insulin_signaling	1.348158e-44	0.001	142
GPCRDB_Other	2.606078e-43	0.001	70
Ribosomal_Proteins	8.182642e-43	0.001	109
Integrin-mediated_cell_adhesion	9.481823e-43	0.001	89
G_Protein_Signaling	2.189569e-41	0.001	86
mRNA processing Reactome	1.481700e-39	0.001	105
G1 to S cell cycle Reactome	6.158349e-39	0.001	65
Electron transport chain	7.287427e-39	0.001	88
Apoptosis	6.909579e-37	0.001	68
Proteasome Degradation	2.345890e-30	0.001	61
Matrix Metalloproteinases	2.799155e-11	0.001	32
Wnt signaling	4.641920e-11	0.001	57
Glycolysis and Gluconeogenesis	1.093909e-10	0.001	47
TGF beta Signaling Pathway	1.504555e-10	0.001	48
Striated muscle contraction	8.382124e-10	0.001	33

Results are ranked according to significance (*t* values from multivariate Westfall-Young analysis).

levels of all seven investigated genes [SREBF1 ($r = -0.618$, $P = 0.000$), FASN ($r = -0.473$, $P = 0.004$), ACACA ($r = -0.507$, $P = 0.002$), LPIN1 ($r = -0.565$, $P = 0.001$), LPL ($r = -0.531$, $P = 0.001$), PPARγ2 ($r = -0.583$, $P = 0.000$), and ADIPOQ ($r = -0.467$, $P = 0.005$)].

In vitro lipogenesis. Given the downregulation of the lipogenic genes in the high VAT/(VAT+SAT) group, we investigated de novo lipogenesis in adipose tissue specimen of 14 subjects (six high vs. eight low VAT/[VAT+SAT] group). The percent increase in lipogenesis after treatment of the samples with insulin tended to be lower (low, 71.1 ± 39.0; high, 31.3 ± 36.7; $P = 0.076$) in the high VAT/(VAT+SAT) group (data not shown).

DISCUSSION

The novel finding of this study is that, in obese adolescents, a high ratio of visceral to subcutaneous fat (i.e., high VAT/[VAT+SAT]) is associated with impaired adipogenesis/lipogenesis as assessed by gene expression and a lower proportion of large adipose cells and higher peak cell diameter as assessed by cell-size distribution analysis.

It should be noted that, although the two groups had a very similar overall degree of adiposity, as well as lean body mass, age, sex, and pubertal stage of development, they had a difference in ethnicity that was close to significant (*P* value in χ^2 test = 0.089). Thus, although we adjusted for this difference in ethnicity when analyzing the differences between the groups, we cannot exclude that some of the differences seen in abdominal fat distribution and adipose cell size are ethnicity related because it is a well known fact that, compared with Caucasians, African Americans have a small visceral and large subcutaneous fat depot (8). To assess directly these potential ethnic differences in adipose cell size and their relations to metabolic alterations, we compared cell-size distribution and gene expression in 11 Caucasian (Caucasian and Caucasian Hispanic) versus 11 African American subjects, pair matched for VAT/(VAT+SAT) ratio, BMI, and age. No differences in cell-size parameters or expression of adipogenic/lipogenic genes were noted, as well as no difference in insulin resistance (data not shown). Thus, we think that the differences found in this study between the two VAT/(VAT+SAT) groups are not accounted for mainly by ethnic differences in cellularity and gene expression of the subcutaneous fat. This is further supported by previous studies (17–19) that showed no difference in the mean diameter of subcutaneous adipocytes from obese African American compared with Caucasian women.

In contrast to our hypothesis of higher insulin sensitivity with lower VAT/(VAT+SAT) ratio, African Americans showed no differences in insulin sensitivity, despite significantly lower VAT/(VAT+SAT) ratio. Further investigations are necessary to unravel the myth of fat distribution and insulin resistance in African American adolescents (manuscript in preparation).

Building on our previous findings (8,13,20), we hypothesized that the particular phenotype of high VAT/(VAT+SAT) might be one of the factors implicated in the pathogenesis of insulin resistance. We consequently focused on the layer of subcutaneous fat, as it is the only one that is relatively easy to biopsy in pediatrics. This depot has been found to be important in rodent models of insulin resistance as well (21,22). Thus, we based the division of the groups on some strong rationale. Nevertheless, even when dividing the groups into insulin sensitive and resistant (data not shown), we found very similar results to those reported when separating the groups by fat ratio.

Adipocyte morphometry. In investigating the adipose cell-size distribution of the subcutaneous fat depot, we found very large adipose cells with diameters in the range of 100–130 μm, but also about half of the cells in the adipose tissue were small, in the 20–50 μm range. While the identity of the latter has been questioned, McLaughlin et al. (6) confirmed the presence of small

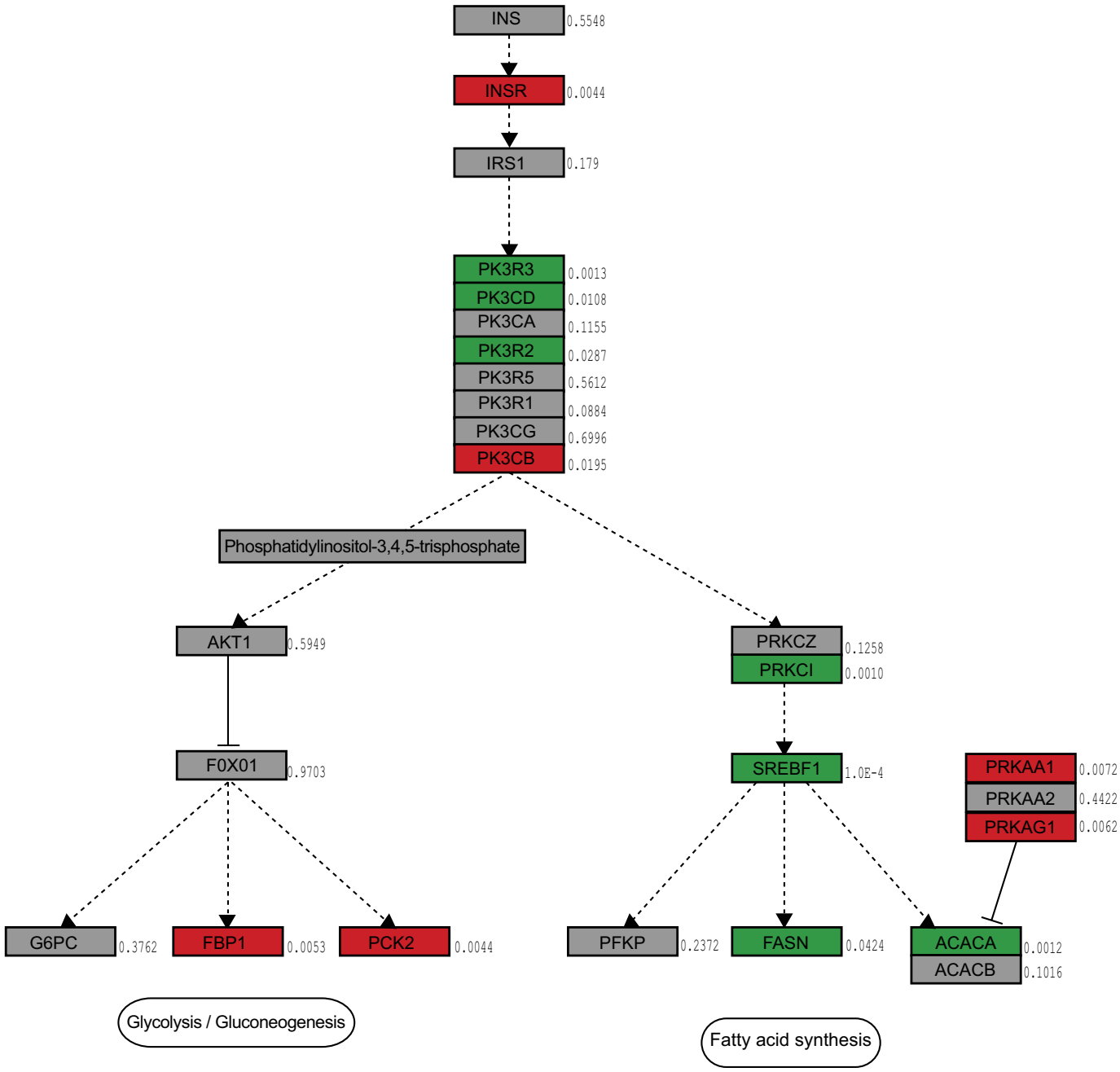

FIG. 3. Diagram of the insulin signaling pathway from PathVisio. Red colored boxes indicate significantly increased expression, whereas green colored boxes indicate a significantly decreased expression in the high versus low VAT/(VAT+SAT) group. Besides the gene boxes, the *P* value is given.

adipose cells in both intact adipose tissue and the osmium-fixed cell preparation by scanning electron microscopy; floatation of isolated adipose cells, prepared by the well established collagenase digestion technique, followed by osmium fixation reproduced the adipose cell-size distributions obtained by direct osmium fixation of samples of the same intact adipose tissue. Moreover, studies of the dynamics of adipose tissue growth in different mouse strains on regular and high-fat diets (16), and in genetically obese Zucker fatty rats (23), demonstrate that the small-cell population detected by the Multisizer plays a systematic role in expanding and contracting fat storage in response to weight gain and loss. RNA extraction and gene expression data in separated small adipose cells from Zucker fatty rats (unpublished data) further support the identification of these small cells. Consistent with our original hypothesis, the subgroup of obese individuals with the high VAT/(VAT+SAT) harbored a greater proportion of the smaller adipose cells, while the diminished population of large cells was characterized by a greater diameter. This relationship suggests hypertrophy of the largest cells, in conjunction with a diminished capacity of the small cells for fat storage. This is supported by our observations that individuals with high VAT/(VAT+SAT) have a tendency of higher plasma levels of free fatty acids and triglycerides and significantly greater quantities of fat stored in their livers. Furthermore, investigating de novo lipogenesis in subcutaneous adipose tissue specimen in a subset of our subjects, the high VAT/(VAT+SAT) group showed a lower percent increase in lipogenesis after treatment of the

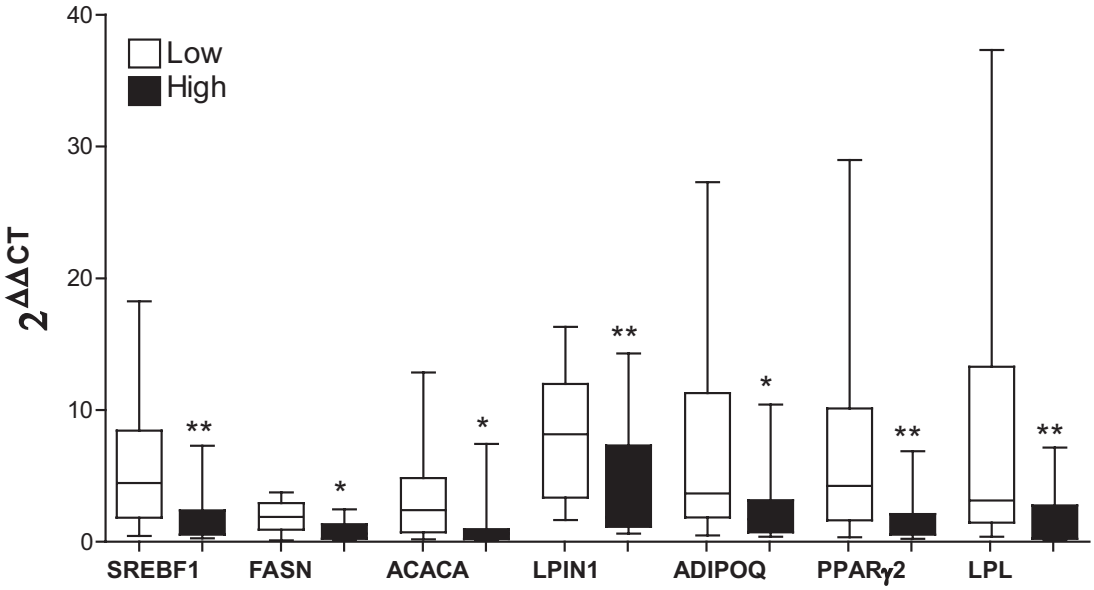

FIG. 4. Box-plots for the expression of SREBF1, FASN, ACACA, LPIN1, ADIPOQ, PPARγ2, and LPL, normalized to the expression of 18S rRNA and based on the expression of a human control adipose tissue ($2^{\Delta\Delta Ct}$). The white boxes represent the means and SD for the group with the low VAT/(VAT+SAT) ratio, and the black boxes represent the means and SD for the group with the high VAT/(VAT+SAT) ratio. The Mann-Whitney test between the two groups was significant at the <0.05 level (*) or at the <0.005 level (**).

samples with insulin. At the molecular level, our findings are further supported by the reduced expression of key regulators of de novo lipogenesis.

Gene-expression analysis. The analysis of our GeneChip data revealed that the insulin signaling pathway is one of the significant differentially regulated pathways between the high and low VAT/(VAT+SAT) groups. This finding goes along with differences in the insulin sensitivity observed between the two groups. An upregulation of AMP-activated kinases (PRKAA1 and PRKAG1) in the high VAT/(VAT+SAT) group leads to downregulation of SREBF1, because SREBF1 is negatively regulated by those kinases. SREBF1 encodes the sterol regulatory element binding protein-1, a transcriptional regulator of ACACA and FASN (24), enzymes that catalyze the committed and rate-limiting steps in cytosolic de novo synthesis of fatty acids. Because ACACA and FASN are regulated primarily at the level of transcription, it is expected that decreases in the activities of these enzymes are reflected by decreases in their mRNA. SREBF1 is also a transcriptional regulator of LPL (24), a rate-limiting enzyme for intravascular hydrolysis of lipoprotein-rich triglyceride particles, which is expressed at high levels in adipose tissue (25), especially of obese, insulin-resistant subjects (26). Furthermore, SREBF1 is able to cause a 3–4-fold increase in the activity of PPARγ through its DNA-binding site and, therefore, stimulates adipogenesis (24). Therefore, downregulation of SREBF1 in the high VAT/(VAT+SAT) group leads to downregulation of ACACA, FASN, LPL, and PPARγ, while PPARγ downregulation further leads to lower expression of ADIPOQ.

Recent studies of rat and human adipose tissue have reported that smaller adipocytes have decreased FASN and LPL enzymatic activities that lead to less de novo synthesis of fatty acids and reduced uptake of lipoproteins for storage (27–29). Ranganathan et al. (30) showed that FASN mRNA in adipose tissue strongly positively correlated to insulin sensitivity and increased after treatment with a PPARγ agonist, pioglitazone. De novo lipogenesis in adipose tissue strongly positively correlates not only with insulin sensitivity (31) but also with BMI and adipocyte cell size (32). Downregulation of lipogenic gene expression in the subcutaneous tissue may therefore be seen as "an impairment of fat cell function," as has been suggested previously by Hoffstedt et al. (31). Thus, larger adipocytes may downregulate lipogenic genes to limit expansion of triglyceride stores and prevent a metabolically detrimental morphology (32). Thus, in the setting of obesity and increased storage demands, if certain individuals are unable to mature a new crop of adipose cells owing to an impairment in differentiation, triglyceride would have to be stored in nonadipose tissues, as reported in insulin-resistant individuals (33). Furthermore, our GeneChip data showed in the group with high VAT/(VAT+SAT) ratio a significantly lower expression of the LPIN1 gene, whose product lipin1 is required for adipocyte differentiation (34). Lipin deficiency causes lipodystrophy, fatty liver, and insulin resistance in mice, whereas adipose tissue lipin overexpression results in increased adiposity but improved insulin sensitivity (35–37). A higher expression of LPIN1 in the low VAT/(VAT+SAT) ratio group is consistent with the role of lipin1 in promoting adipocyte differentiation and lipid accumulation, which could result in a diversion of lipids into adipocytes and away from ectopic sites such as the liver.

Studies in humans have shown the decreased expression of genes related to adipose cell differentiation in the insulin-resistant offspring of patients with type 2 diabetes, compared with insulin-sensitive controls (38), and the upregulation of genes related to adipose cell fat storage in response to the administration of a thiazolidinedione compound that is well known to promote increased insulin sensitivity (39). Similar observations have been reported in Zucker *fa/fa* rats (40). Taken together, our findings and those from adult studies strengthen the hypothesis that, in obese individuals, impairment in adipose cell differentiation is characterized by impaired lipogenesis and a relative reduction in the proportion of large adipose cells, but hypertrophy of the largest cells. The resulting decreased ability to store

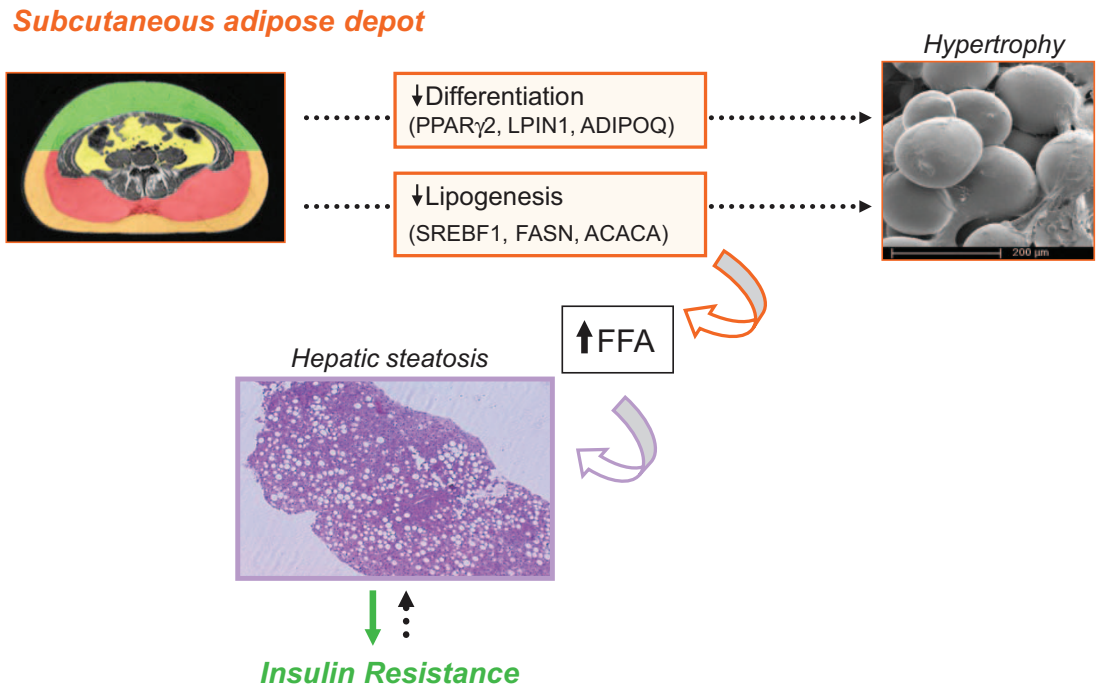

FIG. 5. Schematic representation of the proposed interactions among size of the subcutaneous fat depot, adipogenesis/lipogenesis, and cell size in the subcutaneous adipose tissue, plasma lipid level, hepatic steatosis, and insulin resistance. (A high-quality digital representation of this figure is available in the online issue.)

triglyceride in subcutaneous adipose tissue is likely an important contributor to the development of liver steatosis and insulin resistance.

Our study is limited by its small size. Furthermore, we cannot prove causality, but merely report associations. Note that we are not claiming that small adipose cells differ from large adipose cells with respect to gene-expression and fat metabolism. Rather, we are proposing that differences exist in the homogeneity of the subcutaneous adipose tissue that are associated with quantifiable differences in cell-size distribution. Additional metabolic differences may exist between small and large cells that could contribute to the impact on whole-body metabolism of the differences in cell-size distributions, but such a determination would require further work to separate the cells by size. We adjusted for possible confounding effects of age, sex, and ethnicity. Strengths include the characterization of two similarly obese groups of adolescents who differed in their abdominal fat distribution, the use of state-of-the-art measurements of insulin sensitivity and imaging techniques for assessment of lipid content in liver and muscle, and the use of newer technology to characterize adipose cell-size distribution in a more sophisticated manner, along with measurements of the expression of genes regulating adipogenesis and lipogenesis.

A hypothetical model explaining the cellular and adipogenic profiles of the phenotype high VAT/(VAT+SAT) abdominal fat. The cellularity and, more importantly, the decreased adipogenic and lipogenic profiles profoundly affect the ability of subcutaneous fat to expand (Fig. 5). Lipogenesis in the subcutaneous tissue is turned down, while the amount of plasma free fatty acids rises. The overflow in lipids leads to ectopic fat deposition in the visceral depot and the liver, which in turn leads to an impaired function of the target organ and to insulin resistance.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (R01-HD-40787, R01-HD-28016, and K24-HD-01464) to S.C. and by CTSA Grant No. UL1-RR-0249139 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health; and R01-EB-006494 (Bioimage Suite), and Distinguished Clinical Scientist Awards from the American Diabetes Association (S.C.). T.L., M.B., A.S., and S.W.C. were supported by the Intramural Research Program of the National Institutes of Health, NIDDK. No potential conflicts of interest relevant to this article were reported.

S.C. initiated the concept of the study and designed it together with S.W.C., G.I.S., and R.K. D.N. was the surgeon for the biopsies. T.L. and M.B. performed the Multisizer analyses. A.M.G.C. and E.D. performed the hyperinsulinemic euglycemic clamps. M.S. and B.P. were responsible for collecting the study base from which the 38 subjects were selected. M.E. was responsible for the GeneChip and pathway analyses. R.K., A.S., and M.E. performed the statistical analyses. All authors contributed to the interpretation of the data, and R.K., S.C., and S.W.C. wrote the manuscript.

The authors thank all the volunteers, and Karen Allen, YCCI, Yale University; Adam Thonberg, KECK, Yale University; and Morven Graham, MYSM, Yale University for their skillful help in the study.

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