Original Article

Adipose Tissue Inflammation and Increased Ceramide Content Characterize Subjects With High Liver Fat Content Independent of Obesity

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OBJECTIVE—We sought to determine whether adipose tissue is inflamed in individuals with increased liver fat (LFAT) independently of obesity.

RESEARCH DESIGN AND METHODS—A total of 20 nondiabetic, healthy, obese women were divided into normal and high LFAT groups based on their median LFAT level (2.3 ± 0.3 vs. 14.4 ± 2.9%). Surgical subcutaneous adipose tissue biopsies were studied using quantitative PCR, immunohistochemistry, and a lipidomics approach to search for putative mediators of insulin resistance and inflammation. The groups were matched for age and BMI. The high LFAT group had increased insulin (P = 0.0025) and lower HDL cholesterol (P = 0.02) concentrations.

RESULTS—Expression levels of the macrophage marker CD68, the chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α, and plasminogen activator inhibitor-1 were significantly increased, and those of peroxisome proliferator–activated receptor-γ and adiponectin decreased in the high LFAT group. CD68 expression correlated with the number of macrophages and crown-like structures (multiple macrophages fused around dead adipocytes). Concentrations of 154 lipid species in adipose tissue revealed several differences between the groups, with the most striking being increased concentrations of triacylglycerols, particularly long chain, and ceramides, specifically Cer(d18:1/24:1) (P = 0.01), in the high LFAT group. Expression of sphingomyelinases SMPD1 and SMPD3 were also significantly increased in the high compared with normal LFAT group.

CONCLUSIONS—Adipose tissue is infiltrated with macrophages, and its content of long-chain triacylglycerols and ceramides is increased in subjects with increased LFAT compared with equally obese subjects with normal LFAT content. Ceramides or their metabolites could contribute to adverse effects of long-chain fatty acids on insulin resistance and inflammation. ***Diabetes*** **56:1960–1968, 2007**

Recent studies have shown that the fatty liver is insulin resistant and overproduces many, if not most, of the cardiovascular risk factors associated with the metabolic syndrome (1). Insulin resistance in the liver results in VLDL overproduction, which leads to hypertriglyceridemia and low HDL cholesterol levels (2). The liver, once fatty, also overproduces fibrinogen, von Willebrand factor, C-reactive protein (3), and plasminogen activator inhibitor (PAI)-1 (3,4). Resistance to insulin inhibition of hepatic glucose production basally and postprandially results in hyperglycemia and hyperinsulinemia (1). Abdominal obesity is more common in individuals with a fatty liver than in those without, and intra-abdominal fat correlates with liver fat (LFAT) (5,6). However, the amount of fat in the liver is poorly correlated with subcutaneous obesity (1,5) and predicts the metabolic syndrome (7), type 2 diabetes (8,9), and cardiovascular disease (10) independent of obesity.

Factors regulating LFAT content independent of obesity are poorly understood. Previous studies have shown that adipose tissue in obese subjects is inflamed compared with nonobese subjects (11,12). This inflammation is characterized by an increase in macrophage numbers and expression of macrophage markers such as CD68 (11,12). Expression of insulin-resistant genes and the local production of their protein products by macrophages in adipose tissue are also increased in obese compared with nonobese subjects (13–16). These and other changes may contribute to increased lipolysis and adiponectin deficiency, which characterize adipose tissue of insulin-resistant subjects and may increase LFAT content (17). The stimulus for macrophage accumulation in adipose tissue, on the other hand, is unclear. The first studies comparing obese and nonobese subjects showed macrophage accumulation to be directly related to cell size (11). Recently, Cinti et al. (18) showed that in obese compared with nonobese subjects, macrophages surround dead adipocytes, and they suggested that adipocyte cell death could serve as one stimulus for macrophage infiltration.

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LFAT, liver fat; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein-1α; PAI, plasminogen activator inhibitor; PPAR, proliferator–activated receptor; TNF-α, tumor necrosis factor-α.

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Regarding the causes of adipose tissue inflammation, we have previously shown in two independent studies that the percentage of total fat in diet is positively correlated with LFAT content (19,20). This could alter fatty acid composition of adipose tissue (21). Recent advances in ultra-performance liquid chromatography combined with mass spectrometry methodology and data processing have enabled structural characterization and quantitation of hundreds of lipid species in minute biological samples (22,23). Such methodology has hitherto not been used to study human adipose tissue.

In the present study, we reasoned that fat accumulation in the liver distinguishes between those obese subjects who develop insulin resistance and those who do not. We hypothesized if adipose tissue inflammation is important for fat accumulation in the liver, then adipose tissue should be inflamed in obese subjects who have excess fat in the liver compared with equally obese subjects with normal LFAT content. To examine this, we recruited a group of obese women with a narrow range of BMIs and measured their LFAT content using proton magnetic resonance spectroscopy and body composition using magnetic resonance imaging (MRI) and other techniques. The women were divided into groups with high and normal LFAT content, based on their median LFAT content. The groups were matched with respect to age and body weight. A surgical biopsy of subcutaneous adipose tissue was taken for immunohistochemistry and gene expression studies. Lipidomic analyses were performed using the ultra-performance liquid chromatography combined with mass spectrometry platform.

RESEARCH DESIGN AND METHODS

A total of 20 obese (BMI 30–40 kg/m^2) but otherwise healthy women not using any medications were recruited on the basis of the following inclusion criteria: *1*) aged 18–60 years; *2*) no known major organ system disease determined by history, physical examination, and standard laboratory tests (hemoglobin, sedimentation rate, and electrolytes plasma glucose); *3*) alcohol consumption <20 g/day assessed by a questionnaire (24); *4*) no history of liver diseases; and *5*) stable weight for the last 6 months. All patients had been obese for several years, were physically inactive, and reported consuming a normal Finnish diet.

Patients were studied after an overnight fast. A blood sample was taken for measurement of plasma glucose, serum insulin and C-peptide, serum triglyceride, and total and HDL cholesterol concentrations. A surgical subcutaneous adipose tissue biopsy, yielding ~5 g tissue, was performed under local anesthesia. Part of the biopsy, which was used for gene expression analyses, was immediately frozen in liquid nitrogen and kept at −80°C. Another part of the biopsy was fixed in formalin and embedded in paraffin. Fat cell size was measured by collagenase digestion, as previously described (25). Intrahepatic fat content (percentage LFAT) was determined by magnetic resonance proton spectroscopy, and subcutaneous and abdominal fat areas were measured by MRI. Whole body composition was measured by bioelectrical impedance plethysmography (BioElectrical Impedance Analyzer System, model no. BIA-101A; RJL Systems, Detroit, MI). Waist circumference was measured midway between the spina iliaca superior and the lower rib margin, and hip circumference was measured at the level of the greater trochanters. 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 Helsinki University Central Hospital and the Karolinska Institutet.

Total RNA and cDNA preparation. Frozen adipose tissue (50–150 mg) was homogenized in 2 ml RNA STAT-60 (Tel-Test, Friendswood, TX), and total RNA was isolated according to the manufacturer's instructions, as described. After DNase treatment, RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit; Molecular Probes, Eugene, OR). The quality of RNA was analyzed by Agilent Bioanalyzer 2100 (Agilent Technologies). Average yields of total RNA were 3 ± 1 μg per 100 mg adipose tissue wet wt and did not differ between the groups. Isolated RNA was stored at −80°C until the quantification of the target mRNAs. A total of 0.1 μg RNA was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Paisley, U.K.) and oligo $(dT)_{12\text{-}18}$ primer.

Quantification of gene expression. mRNA expression of specific genes was quantified by real-time PCR using the ABI 7000 Sequence Detection System instrument and software (Applied Biosystems). cDNA synthesized from 15 ng total RNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan Gene Expression Assays; Applied Biosystems) in a final volume of 25 μl. The assays used included: Hs00154355_m1 for CD68, Hs00234140_m1 for CCL2, Hs00234142_m1 for CCL3, Hs00605917_m1 for adiponectin, Hs00234592_m1 for peroxisome proliferator–activated receptor (PPAR)-γ, Hs00194153_m1 for 11βHSD1, Hs00174131_m1 for interleukin 6, Hs00174128_m1 for tumor necrosis factor (TNF)-α, Hs00167155_m1 for PAI-1, Hs00609415_m1 for SMPD1, Hs00906924_g1 for SMPD2, Hs00218713_m1 for SMPD3, Hs99999910_m1 for TBP (TATA-box binding protein), and Hs99999902_m1 for RPLP0 (ribosomal protein large P0). All samples were run in duplicate. Relative expression levels were determined using a five-point serially diluted standard curve, generated from cDNA of human adipose tissue. Gene expression was expressed in arbitrary units and normalized relative to the housekeeping genes RPLP0 and TBP to compensate for differences in cDNA loading. The average of these two values was used for normalization.

Immunohistochemistry. Subcutaneous biopsies from all subjects were used for immunohistochemical staining for the study of macrophages and necrotic adipocytes. CD68 was used as a marker for macrophages, and perilipin was used as a negative marker for necrotic adipocytes, as previously described by Cinti et al. (18). Staining was performed using a standard protocol on sections from formalin-fixed paraffin-embedded tissue blocks. Serial sections were microwave-treated in 10 mmol/l citrate buffer (pH 6.0) and then incubated for 1 h at room temperature with primary antibodies, mouse monoclonal anti-CD68 (Novocastra Laboratories, Newcastle upon Tyne, U.K.), polyclonal guinea pig anti-perilipin (Acris, Hiddenhausen, Germany), or with a mouse monoclonal isotypic control (Abcam, Cambridge, U.K.) for CD68. After rinsing in PBS buffer containing 0.25% Triton X-100 (pH 7.2), sections were incubated with secondary biotinylated goat anti-mouse (Dako cytomation; Dako, Glostrup, Denmark) or biotinylated goat anti–guinea pig (Abcam) antibodies. Avidin-biotin peroxidase complexes (Vector Laboratories, Burlingame, CA) were added followed by visualization with 3.3′-diaminobenzidine tetrachloride (Vector). All sections were counterstained with Harris hematoxylin (Histolab, Göteborg, Sweden). For each subject, the numbers of macrophages (identified as CD68+ cells) and crown-like structures within the entire section were counted by two independent observers using a light microscope and normalized for the total section area. A crown-like structure was defined as a perilipin-free adipocyte surrounded by at least three macrophages (18). Measurement of total section area and average adipocyte area (in arbitrary units) was performed using the GNU Image Manipulation Program 2.2 (GIMP 2.2).

Measurement of monocyte chemoattractant protein (MCP)-1 expression in adipose tissue. A frozen sample of human subcutaneous adipose tissue (100–430 mg) was homogenized in lysis buffer (26). The homogenate was centrifuged for 30 min (+4°C, 14,000 rpm) and the supernatant stored at −80°C until measurement of the MCP-1 concentration using the Human CCL2/MCP-1 Immunoassay kit (Quantikine; R&D Systems, Minneapolis, MN) and the Bio Assay Reader HTS 7000 Plus (Perkin Elmer, Norwalk, CT). Total protein was measured using the BC Assay-protein quantitation kit (Uptima Interchim, Montlucan, France).

Lipidomics/adipose tissue sample preparation. Approximately 20 mg adipose tissue was weighed, and 20 μl of an internal standard mixture and 40 μl NaCl (0.9%) were added to the sample. Lipids were extracted from the samples with 200 μl chloroform:methanol (2:1) solvent, and the tissue was homogenized with a glass rod. After vortexing for 2 min and incubating for 1 h at room temperature, the lower layer (~100 μl) was separated by centrifugation at 10,000 rpm for 3 min at room temperature. Labeled standard mixture (20 μl) was added to the lipid extract. Internal and external standards are listed in the supplemental materials (found in the online appendix [available at http://dx.doi.org/10.2337/db07-0111]).

Lipidomic analysis. Lipid extracts were analyzed on a Q-ToF Premier mass spectrometer (Waters, Milford, MA) combined with an Acquity ultra-performance liquid chromatography. The column was an Acquity ultra-performance liquid chromatography bridged ethyl hybrid C18 10 × 50 mm with 1.7-μm particles and was kept at 50°C. The binary solvent system A included water (1% 1M NH_4Ac, 0.1% HCOOH), and solvent system B included LC/MS grade (Rathburn) acetonitrile/isopropanal (5:2, 1% 1M NH_4Ac, 0.1% HCOOH). The gradient started from 65% A/35% B, reached 100% B in 6 min, and remained there for the next 7 min. The total run time, including a 5-min reequilibration step, was 18 min. The flow rate was 0.2 ml/min, and injection volume was 1 μl. The temperature of the sample

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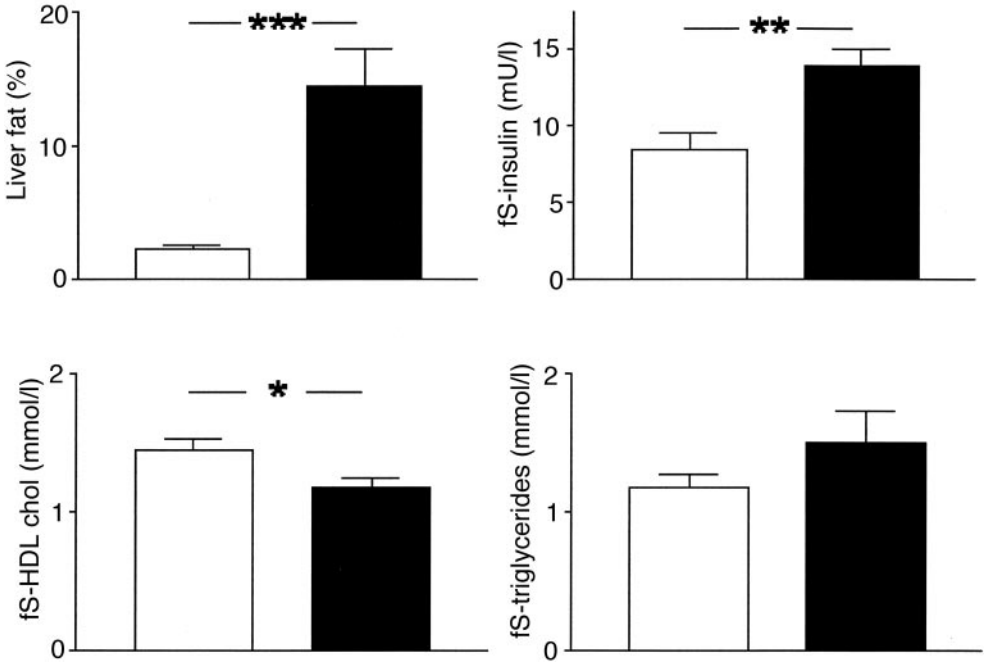

FIG. 1. LFAT percentage and fasting serum (fS) insulin, fS-HDL cholesterol, and fS-triglyceride concentrations in women with normal (□) and high (■) LFAT. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

organizer was set at 10°C. Lipid profiling was carried out using positive ion mode. The data were collected at mass range of charge/mass ratio 300–2,000, with a scan duration of 0.2 s. The source temperature was set at 120°C, and nitrogen was used as desolvation gas (800 l/h) at 250°C. The voltages of the sampling cone and capillary were 39 V and 3.2 kV, respectively. Reserpine (50 μg/l) was used as the lock spray reference compound (5 μl/min), with a 10-s scan frequency.

The obtained data were converted into netCDF file format using Dbridge software from MassLynx (Waters). The converted data were processed using MZmine software version 0.60 (22). Lipids were identified based on their retention time and mass/charge ratio using our in-house built lipid database as previously described (27). All the identified lipids were quantified by normalizing with corresponding internal standards. Sphingomyelins were normalized with GPCho(17:0/17:0) internal standard.

Analytical procedures. Plasma glucose concentrations were measured in duplicate with the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Serum-free insulin concentrations were measured using the Auto-DELFIA kit from Wallac (Turku, Finland) and C-peptide concentrations by radioimmunoassay. Serum total cholesterol, HDL cholesterol, and triglyceride concentrations were measured with enzymatic kits from Roche Diagnostics, and serum free fatty acids were measured with an enzymatic kit from Wako using an autoanalyzer (Roche Diagnostics Hitachi 917; Hitachi, Tokyo, Japan). The concentration of LDL cholesterol was calculated using the Friedewald formula.

Statistical analysis. Statview (SAS Institute, Cary, NC) software was used to perform the statistical analysis. Physical and biochemical characteristics of the study subjects were analyzed using nonparametrical methods. Groups were compared using the Mann-Whitney test. All correlations were performed using Spearman's rank correlation. Differences in gene expression and lipidomics data were compared by unpaired t test after logarithmical transformation of variables (gene expression data only) with non–normal distribution. Statistical significance was assigned to a value of $P < 0.05$. Data are presented as mean ± SEM.

TABLE 1
Physical and biochemical characteristics of the study subjects divided into normal and high LFAT groups

	Normal LFAT	High LFAT	Significance*
n	10	10	—
Age (years)	44 ± 3	37 ± 2	NS
Body weight (kg)	98 ± 2	98 ± 3	NS
BMI (kg/m^2)	35.4 ± 1.1	36.7 ± 0.8	NS
Waist-to-hip ratio	0.98 ± 0.06	0.97 ± 0.03	NS
Whole body fat (%)	39.7 ± 1.0	37.1 ± 1.2	NS (0.08)
Intra-abdominal fat (cm^3)	1,631 ± 167	1,979 ± 261	NS
Subcutaneous fat (cm^3)	7,407 ± 333	6,644 ± 526	NS
Fat mass (kg)	38.7 ± 1.3	36.7 ± 1.9	NS
LFAT (%)	2.3 ± 0.3	14.4 ± 2.9	0.0002
Fasting plasma glucose (mmol/l)	5.1 ± 0.2	5.5 ± 0.2	NS
A1C (%)	5.4 ± 0.1	5.7 ± 0.2	NS
Fasting serum insulin (mU/l)	8.4 ± 1.0	13.9 ± 1.2	0.0025
Fasting serum C-peptide (nmol/l)	0.88 ± 0.07	1.12 ± 0.10	NS (0.08)
Fasting serum LDL cholesterol (mmol/l)	2.6 ± 0.2	3.0 ± 0.2	NS
Fasing serum HDL cholesterol (mmol/l)	1.5 ± 0.1	1.2 ± 0.1	0.02
Fasting serum triglycerides (mmol/l)	1.2 ± 0.1	1.5 ± 0.2	NS
Fasting serum free fatty acids (μmol/l)	669 ± 101	606 ± 76	NS

Data are means ± SEM. *Exact P values are shown in parentheses for significances in the 0.05–0.10 range.

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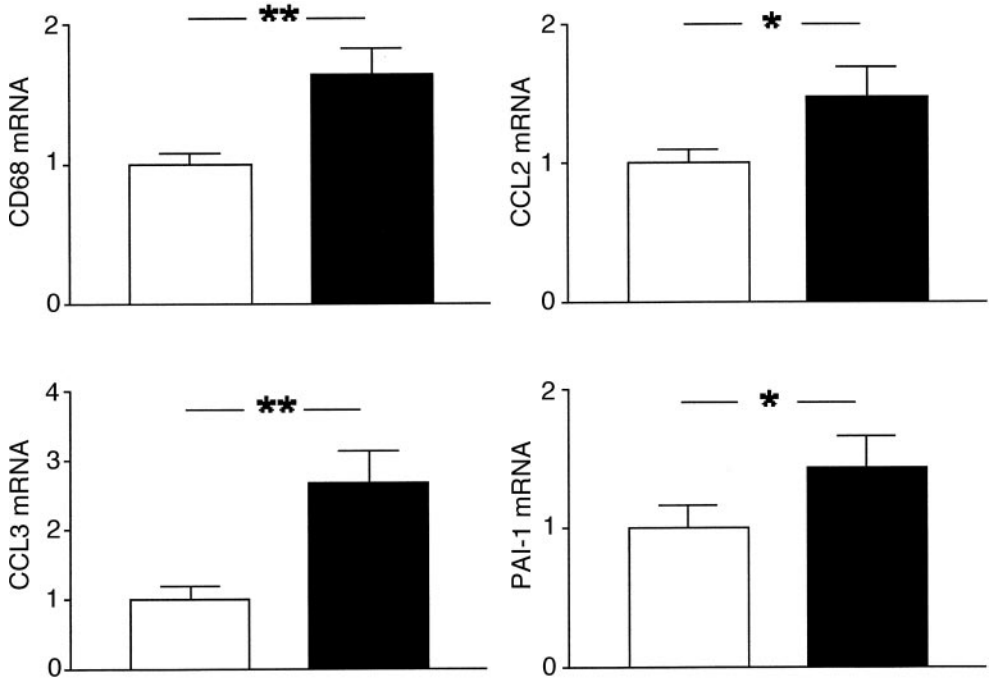

FIG. 2. mRNA expression of CD68, CCL2, CCL3, and PAI-1 in subcutaneous adipose tissue in women with normal (□) and high (■) LFAT. Expression is in arbitrary units normalized for housekeeping genes RPLP0 and TBP. *$P < 0.05$, **$P < 0.01$.

RESULTS

The 20 women were divided into two groups according to their LFAT content: normal LFAT ($n = 10$, range 1–3.5%, mean 2.3 ± 0.3%) and high LFAT ($n = 10$, range 6–34%, mean 14.4 ± 2.9%). Characteristics of the normal and high LFAT groups are listed in Table 1. The groups were similar, with respect to age, sex, BMI, and subcutaneous and intra-abdominal fat masses. Fasting serum insulin concentrations were significantly higher and HDL cholesterol concentrations lower in the high LFAT group compared with the normal LFAT group (Table 1 and Fig. 1).

Expression in subcutaneous adipose tissue of the macrophage markers CD68, CCL2 (MCP-1) and CCL3 (macrophage inflammatory protein [MIP]-1α), and PAI-1 was significantly higher in the high LFAT group compared with the normal LFAT group (Table 2 and Fig. 2). MCP-1 protein expression in adipose tissue correlated with gene expression ($r = 0.53$, $P = 0.023$). Expression of adiponectin and PPAR-γ was significantly lower in the high LFAT group than in the normal LFAT group (Table 2). Expression of 11βHSD1, interleukin 6, and TNF-α did not differ significantly between the groups, although TNF-α tended to be increased in the high LFAT group (Table 2). Expression of the housekeeping genes RPLP0 and TBP did not differ between the groups (RPLP0: 1.9 ± 0.3 vs. 2.1 ± 0.2, NS, and TBP: 4.0 ± 0.5 vs. 4.0 ± 0.4, NS, for normal vs. high LFAT groups, respectively). Expression of the macrophage marker CD68 correlated significantly with LFAT ($r = 0.67$, $P = 0.0035$) but not with BMI ($r = 0.10$, NS) (Fig. 3*B* and *C*).

TABLE 2
Relative gene expression levels in subcutaneous adipose tissue in normal and high LFAT groups

Gene	Normal LFAT	High LFAT	*P*
CD68	1.9 ± 0.1	3.2 ± 0.4	0.003
CCL2	0.35 ± 0.03	0.52 ± 0.08	0.05
CCL3	0.36 ± 0.07	1.0 ± 0.2	0.002
Adiponectin	1.0 ± 0.1	0.9 ± 0.1	0.04
11βHSD1	5.7 ± 1.0	5.6 ± 0.9	NS
IL6	0.12 ± 0.03	0.12 ± 0.02	NS
TNF-α	1.5 ± 0.3	2.4 ± 0.8	NS (0.12)
PAI-1	2.8 ± 0.5	4.0 ± 0.6	0.03
PPAR-γ	1.1 ± 0.09	0.9 ± 0.05	0.04
SMPD1	1.5 ± 0.1	1.9 ± 0.1	0.01
SMPD2	1.1 ± 0.1	1.3 ± 0.1	NS (0.08)
SMPD3	1.3 ± 0.1	1.8 ± 0.2	0.05

Data are means ± SEM. Expression of each gene is normalized to the expression of housekeeping genes TBP and RPLP0.

Adipocyte cell size. The average adipocyte cross-sectional area in the sectioned tissue and fat cell size measured by collagenase digestion did not differ between the normal and high LFAT groups (2,067 ± 109 vs. 2,118 ± 207 arbitrary units, NS, for cross-sectional area and 25.2 ± 0.8 vs. 24.9 ± 1.0 μm for fat cell size, NS).

Immunohistochemistry. Positive staining for the macrophage marker CD68 was seen in 19 of 20 adipose tissue biopsies. The total number of macrophages per section area did not differ between the normal and high LFAT groups (22 ± 6 vs. 37 ± 10, NS, for normal vs. high LFAT groups, respectively) but did correlate significantly with the mRNA expression of CD68 in adipose tissue in all subjects ($r = 0.53$, $P = 0.02$) (Fig. 3*A*). Some macrophages were arranged in crown-like structures (Fig. 4) around necrotic perilipin-free adipocytes (Fig. 5). The number of crown-like structures per section area correlated with borderline significance with the mRNA expression of CD68 ($r = 0.44$, $P = 0.057$).

Lipidomics of adipose tissue. Lipids were analyzed from subcutaneous adipose tissue of normal and high LFAT subjects. A total of 154 lipid molecular species were identified. The differences were dominated by increased ceramides, sphingomyelins, ether phospholipids, and triacylglycerols. No specific trend was observed for ester-linked phospholipids or diacylglycerols. Specifically, all

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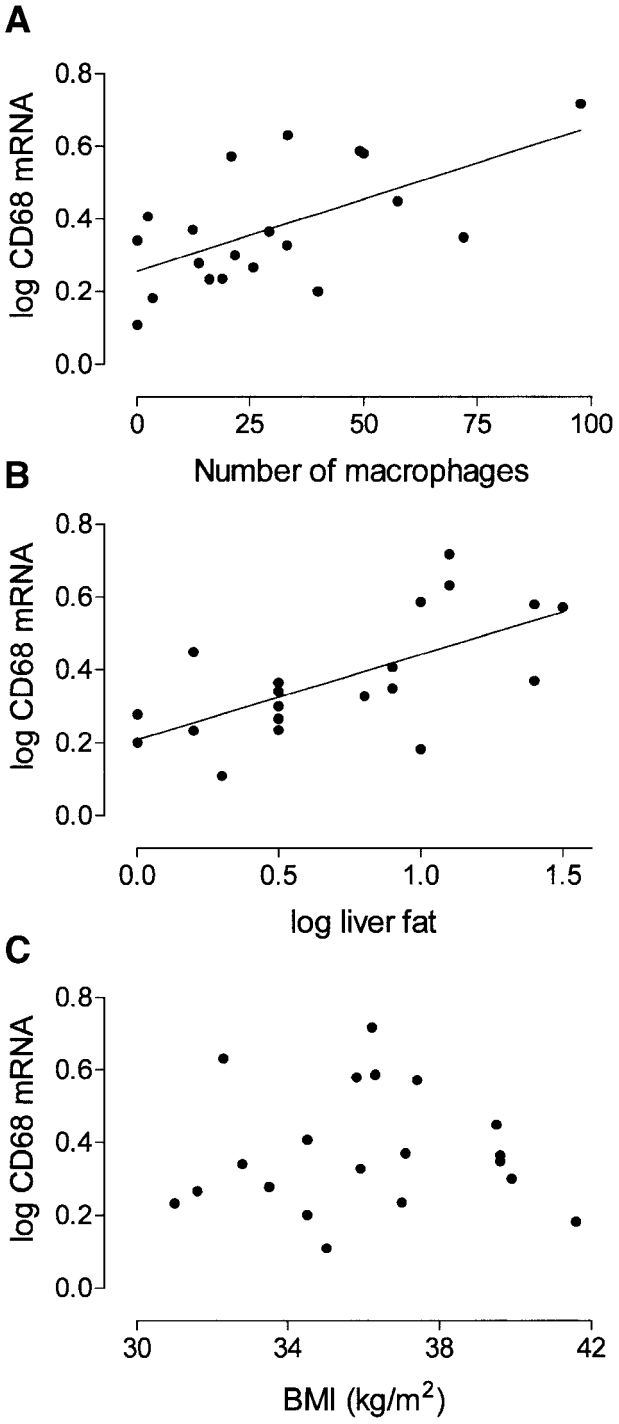

FIG. 3. The relationship between CD68 expression in adipose tissue and the number of macrophages (*A*) ($r = 0.53$, $P = 0.02$), LFAT (*B*) ($r = 0.67$, $P = 0.0035$), and BMI (*C*) ($r = 0.10$, NS).

three identified ceramide molecular species were increased in the high LFAT group, with the most abundant species Cer(d18:1/24:1) being increased 1.5-fold ($P < 0.01$) (Fig. 6*A*). The sphingomyelin molecular species were proportionally increased, although the levels of the most abundant sphingomyelin molecular species SM(d18:1/16:0) did not differ between the normal and high LFAT groups. Expression levels in adipose tissue of the sphingomyelinases SMPD1 and SMPD3 were significantly greater in the high LFAT group compared with the normal LFAT group (Table 2). Most of the 95 identified triacylglycerol species were increased in the high LFAT group. The degree of difference was positively correlated with the triacylglycerol carbon chain length (Fig. 6*B*). No such trend was observed for the degree of fatty acid saturation. Several of the most elevated triacylglycerol species contained an odd number of total fatty acid chain carbons (Fig. 6*B*).

DISCUSSION

This is the first study that has sought to determine whether increased LFAT, independent of obesity, is associated with adipose tissue inflammation as determined by quantitative PCR, immunohistochemistry, and lipidomics approaches. It is also the first study to use a lipidomics approach to analyze adipose tissue in human subjects. We found that several of the changes previously attributed to obesity characterize equally obese subjects who differ with respect to LFAT content. High LFAT was associated with increased expression of CD68, CCL2 (MCP-1), CCL3 (MIP-1α), and PAI-1 and decreased expression of PPAR-γ in adipose tissue. Average fat cell sizes were similar between the groups when measured using both collagenase digestion and morphometry. CD68 expression in adipose tissue correlated with the number of macrophages and crown-like structures when adipose tissue was analyzed by immunohistochemistry. We also found, as has previously been found in obese subjects compared with lean subjects by Cinti et al. (18), that the crown-like structures surrounded perilipin-negative adipocytes. The lipidomics analysis revealed in the high LFAT group an increase in the sphingolipid ceramide and in long-chain triacylglycerols. Although this human study cannot prove cause and effect, ceramide synthesis is stimulated by long-chain fatty acids, and ceramides can induce both inflammation and insulin resistance (28).

We divided the study subjects according to their median LFAT into two groups. These groups were matched with respect to age, sex, BMI, waist circumference, and amounts of intra-abdominal and subcutaneous fat measured with MRI. The highest LFAT content in the normal LFAT group was 3.5% and the lowest content in the high LFAT group 6%. In the Dallas Heart Study (29), in which LFAT was measured in 2,349 subjects using proton magnetic resonance spectroscopy, the upper limit of normal for LFAT was defined as 5.56%. This upper limit for LFAT, when measured with spectroscopy, is similar to that which we have found to correspond to the upper limit of S-alanine aminotransferase in women and men (5). Based on these data, the normal LFAT group in the present study indeed had normal LFAT content, and the high LFAT group had increased LFAT. In keeping with previous data in weight-matched groups (1,30), the subjects with increased LFAT had signs of insulin resistance (higher insulin and lower HDL cholesterol concentrations) compared with those with normal LFAT (Table 1).

Expression of CD68, CCL2 (MCP-1) and CCL3 (MIP-1α), and PAI-1 were significantly increased and PPAR-γ significantly decreased in the high compared with the normal LFAT group (Table 2). Previous studies have documented increases in gene and protein expression of each of these genes in obese subjects compared with nonobese subjects (31–35). The increased expression of MCP-1 is of particular interest because overexpression of CCL2 (MCP-1) in adipose tissue in mice is sufficient to induce insulin resistance and macrophage infiltration and increase triglyceride content (36). CCR2 (CCL2 receptor) deficiency has opposite effects (37). We did not attempt to determine

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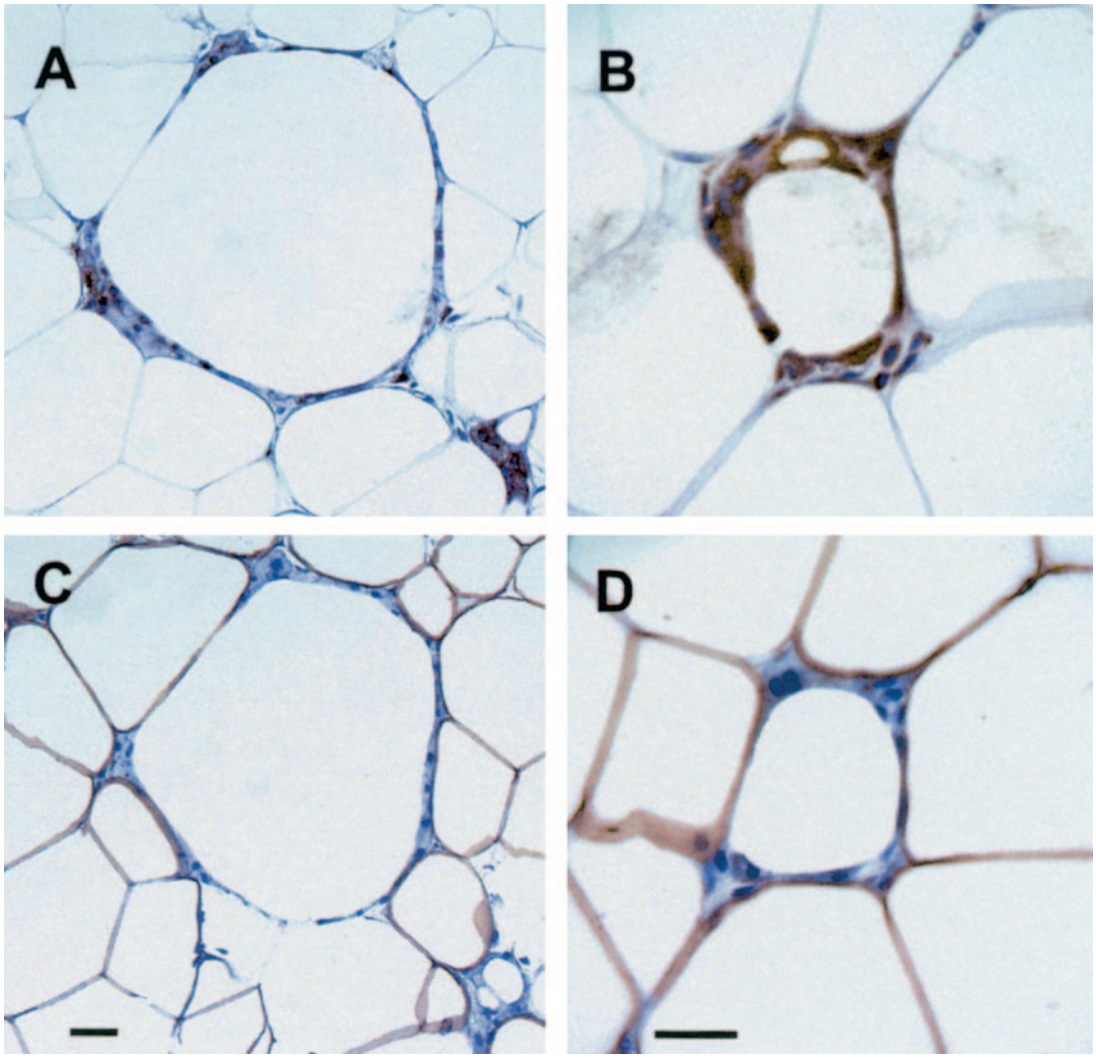

FIG. 4. Representative pictures showing crown-like structures in subcutaneous adipose tissue from an obese woman. Immunohistochemical staining for macrophage specific CD68 (*A* and *B*) is colored brown. *C* and *D*: Serial sections of *A* and *B*, respectively, were stained for perilipin (brown). All sections were counterstained with hematoxylin (colored blue). Scale bar: 20 μm.

the cellular origin of MCP-1, as several studies have consistently shown macrophages to be the main source of this chemokine in human adipose tissue (11,12,38). CCL3 (MIP-1α) is a monokine involved in the recruitment and activation of polymorphonuclear leukocytes (39). In the present study, PPAR-γ expression was significantly lower in the high than in the normal LFAT group. Ablation of adipocyte PPAR-γ expression in mice has been shown to lead to adipocyte necrosis and infiltration of inflammatory cells in adipose tissue (40). Therefore, the current observation of reduced adipose tissue PPAR-γ expression in the high LFAT group may have contributed to the greater levels of inflammation in the adipose tissue of these subjects.

Previous studies by Cancello et al. (13) and Cinti et al. (18) have demonstrated that the number of macrophages is increased in obese subjects compared with nonobese subjects. Furthermore, several studies have shown that macrophages form crown-like structures (11,12,38), which surround perilipin-negative adipocytes (18). The number of crown-like structures was increased in both obese mice and humans. The present data confirm the presence of these structures in human subcutaneous adipose tissue. The finding of increased macrophage infiltration independent of obesity is novel. The crown-like structures were found to surround perilipin-negative adipocytes. Perilipin, a protein involved in the regulation of lipolysis, is located at the interface between the cytosol and the triacylglycerol droplets of adipocytes (41). Cinti et al. (18) found negative perilipin staining in adipocytes, the membranes of which were disrupted and engulfed by macrophages when studied by electron microscopy. Negative perilipin staining was used as a marker of adipocyte death in the current study.

Fat cell size has been shown to correlate with obesity in multiple studies (42) and was also closely correlated with the degree of obesity in studies showing macrophage infiltration in adipose tissue (11). In a study by Cinti et al. (18), cell death correlated with mean adipocyte size. In $HSL^{-/-}$ mice, adipocyte cell size was markedly increased but body fat mass similar to that of wild-type mice. In these $HSL^{-/-}$ mice, adipose tissue was infiltrated with macrophages, implying that increased cell size rather than overall obesity is a trigger for macrophage infiltration (18). In the present study in equally obese groups, we found no difference in average fat cell size.

Regarding the mechanisms underlying the increase of ceramides, several factors could be involved. First, dietary

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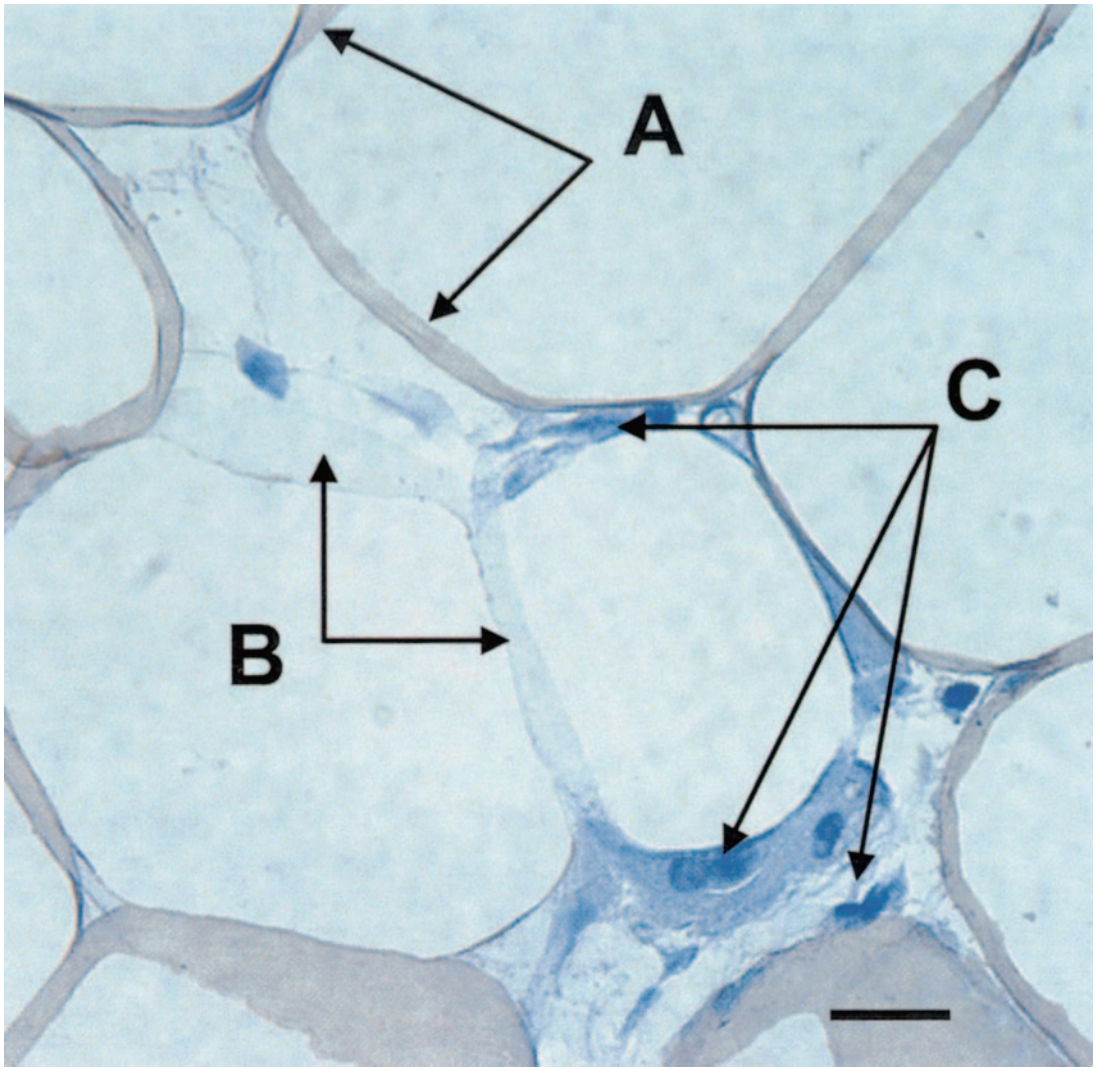

FIG. 5. Light microscopy of subcutaneous adipose tissue from an obese woman stained for perilipin (brown) showing dead (perilipin-free) adipocytes surrounded by macrophages (blue) forming a crown-like structure. The blue-colored nuclei (labeled C) stained positive for the macrophage-specific marker CD68 on a serial section (not shown). *A* arrows, perilipin-positive staining; *B* arrows, perilipin-negative staining; and *C* arrows, macrophages. Scale bar: 20 μm.

supplementation has been shown to increase plasma sphingomyelin concentrations (28). Sphingolipids are particularly abundant in dairy products, suggesting that their intake was greater in the high rather than the normal LFAT group. This theory is supported by the observation in the lipidomics analysis that the most upregulated triacylglycerol species contained odd numbers of total fatty acid carbons (Fig. 6*B*), implying that the concentration of odd-chain fatty acids was increased. Such fatty acids are likely to include fatty acids of exogenous origin specific for ruminant fat, namely 15:0 and 17:0, which are biological markers of dairy fat intake (43). These data are in agreement with previous data demonstrating that a relative high-fat intake correlates with increased LFAT both in type 2 diabetic patients (19) and in monozygotic twins discordant for obesity (20) and that LFAT content can be increased by a high-fat compared with low-fat diet in humans (44). Thus, ceramides could be mediators of the adverse metabolic effects of high-fat diets.

Increased de novo biosynthesis or hydrolysis of sphingomyelin via activation of sphingomyelinase(s) could also have contributed to the increase in ceramide content in the high LFAT group (28). The initial rate-limiting step of de novo biosynthesis of ceramides is strongly dependent on long-chain fatty acid availability (28), which was, based on the lipidomics analysis of adipose tissue, significantly increased in the high LFAT compared with the normal LFAT group. Finally, although enzymatic activities were not determined, expression levels of SMPD1 (acid sphingomyelinase) and SMPD3 (neutral sphingomyelinase 2) were significantly increased in the high compared with the normal LFAT group. Both SMPD1 and SMPD3 are increased by several cytokines including TNF-α (45). In the present study, TNF-α expression in adipose tissue was 1.6-fold greater in the high LFAT group, but this was not statistically significant ($P = 0.12$), possibly due to a type II error. Interestingly, sphingomyelinase expression is heavily increased in the arterial intima (46), and the ceramide content of LDL in the arterial wall is higher than that of native LDL in the plasma (47). Thus, the changes in inflamed adipose tissue containing macrophages bear some resemblance to changes characteristic of atherosclerosis in the arterial wall.

In conclusion, we demonstrated that adipose tissue is infiltrated with macrophages and that its content of long-chain triacylglycerols and ceramides is increased in sub-

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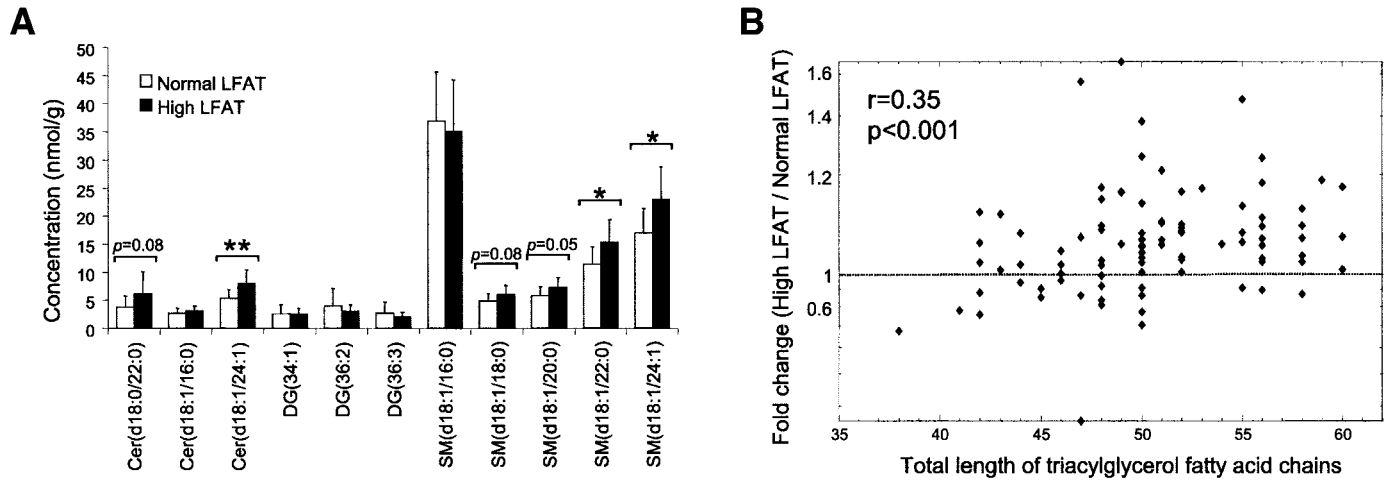

FIG. 6. Lipidomics profiling of subcutaneous adipose tissue. *A*: Levels of detected ceramide, diacylglycerol, and selected sphingomyelin species. *$P < 0.05$, **$P < 0.01$. *B*: Triacylglycerol carbon chain length correlates with the fold change between normal and high LFAT groups. Spearman correlation was applied to calculate r, with P value testing the hypothesis of no correlation against the alternative that there is a nonzero correlation using the large sample approximation.

jects with increased LFAT compared with equally obese subjects with normal LFAT content. Ceramides could mediate adverse effects of long-chain fatty acids and induce both insulin resistance and inflammation, although the present study does not prove cause and effect.

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