Original Article

Increased Infiltration of Macrophages in Omental Adipose Tissue Is Associated With Marked Hepatic Lesions in Morbid Human Obesity

Raffaella Cancello,[1,2,3] Joan Tordjman,[1,2,3] Christine Poitou,[1,2,3] Gaël Guilhem,[1,2,3] Jean Luc Bouillot,[4] Danielle Hugol,[5] Christiane Coussieu,[6] Arnaud Basdevant,[1,2,3] Avner Bar Hen,[7] Pierre Bedossa,[8,9] Michèle Guerre-Millo,[1,2,3] and Karine Clément[1,2,3]

In human obesity, white adipose tissue (WAT) is enriched in macrophages. How macrophage infiltration in WAT contributes to the complications of obesity is unknown. This study tested the hypothesis that recruitment of macrophages in omental WAT is associated with hepatic damage in obese patients. Paired biopsies of subcutaneous and omental WAT and a liver biopsy were collected during gastric surgery in 46 obese women and 9 obese men (BMI 47.9 ± 0.93 kg/m^2). The number of HAM56+ macrophages in WAT was quantified microscopically, and correlations with clinical and biological parameters and histological liver pathology were investigated. There were twice as many macrophages in omental as in subcutaneous WAT ($P < 0.0001$). After adjustment for age, omental WAT macrophage infiltration was correlated to fasting glucose and insulin, quantitative insulin sensitivity check index, triglycerides, aspartate aminotransferase (AST), and γ-glutamyltranspeptidase. We propose an easy equation to estimate the amount of macrophages in omental WAT. Increased macrophage accumulation specifically in omental WAT was associated with hepatic fibroinflammatory lesions ($P = 0.01$). The best predictive model for the severity of hepatic damage includes adiponectinemia, AST, and omental WAT macrophages. These data suggest that the presence of macrophages in omental WAT participates in the cellular mechanisms favoring hepatic fibroinflammatory lesions in obese patients. *Diabetes* 55:1554–1561, 2006

From the [1]Institut National de la Santé et de la Recherche Médicale (INSERM), U755 Nutriomique, Paris, France; the [2]Pierre and Marie Curie-Paris 6 University, Faculty of Medicine, Les Cordeliers, IFR58, Paris, France; the [3]Nutrition Department, AP-HP, Hôtel-Dieu Hospital, Paris, France; the [4]Surgery Department, AP-HP, Hôtel Dieu Hospital, Paris, France; the [5]Anatomo-Pathology Department, AP-HP, Hôtel Dieu Hospital, Paris, France; the [6]Biochemistry Department, AP-HP, Hôtel Dieu Hospital, Paris, France; the [7]LIM and BIO, Paris 13 University, Bobigny, France; the [8]Pathology Department, AP-HP Beaujon Hospital, Clichy France; and the [9]National Center for Scientific Research, Unité Mixte de Recherche 149, Paris, France.

Address correspondence and reprint requests to Prof. Karine Clément, INSERM, U755 Nutriomique, Service de Nutrition, Hôtel-Dieu, 1 Place du Parvis Notre-Dame, 75004 Paris, France. E-mail: karine.clement@htd.ap-hop-paris.fr.

Received for publication 31 January 2006 and accepted in revised form 2 March 2006.

R.C. and J.T. contributed equally to this work.

AST, aspartate aminotransferase; γGT, γ-glutamyltranspeptidase; NAFLD, nonalcoholic fatty liver disease; QUICKI, quantitative insulin sensitivity check index; TBS-TC, Tris-buffered saline/Tween 20/casein 0.02 mol/l solution; TNF, tumor necrosis factor; WAT, white adipose tissue.

DOI: 10.2337/db06-0133

Macrophages are mononuclear phagocytes involved in immunological and inflammatory processes, whose specific function is to provide an immediate defense against foreign elements such as pathogens and dead cells. Resident macrophages are found in virtually all tissues (1). However, the presence of macrophages in white adipose tissue (WAT) has gone almost unnoticed, except for specific experimental conditions leading to adipose cell death in mice (2). It is now established that macrophages are scarce in the WAT of normal-weight individuals, but they increase markedly in animal models and in human obesity (3–6). Transplantation studies in mice suggest that these macrophages derive mostly from the bone marrow (3) rather than from preadipocyte differentiation toward macrophage lineage (7,8).

The causes and consequences of macrophage recruitment in WAT are poorly understood. Substantial infiltration of inflammatory cells around necrotic-like adipocytes has been reported in experimental models of adipose cell death (2,9). In humans, the presence of CD68-positive macrophages in direct contact with mature adipocytes has been noted on adipose tissue slides (10). More recently, arrangement of macrophages in a "crown" around single adipocytes exhibiting features of necrosis has been reported in obese subjects (6,11). These observations suggest that WAT macrophages have a phagocytic activity toward deficient, stressed, or dead-like adipocytes. This function could help control the mass expansion and integrity of WAT. Because of their capacity to produce inflammatory factors, WAT macrophages may also contribute to the low-grade chronic inflammation observed in human obesity. This hypothesis is supported by our results showing that a weight loss–induced improvement of systemic inflammation is associated with a reduction in macrophage infiltration and improvement of the inflammatory profile in subcutaneous WAT (6,12). It has been suggested that WAT macrophages may play a role in the etiopathogenesis of obesity comorbidities. This is supported by results showing that macrophage accumulation in WAT precedes high-fat diet–induced hyperinsulinemia in mice, suggesting a causative link between WAT macrophages and insulin resistance (4). Similarly, deletion of CCR2 (chemokine receptor-2) lowered the macrophage content of epididymal WAT and improved systemic glucose homeostasis and insulin sensitivity in obese mice

TABLE 1
Clinical and biological parameters of 55 morbidly obese subjects

Phenotype	Whole population	Liver subset
n	55	39
F/M	46/9 (83)	31/8 (80)
Age (years)	39.5 ± 1.44	38.3 ± 1.63
BMI (kg/m^2)	47.9 ± 0.93	47.8 ± 1.15
Glucose homeostasis		
Glucose (mmol/l)	6.15 ± 0.29	6.24 ± 0.33
Insulin (μU/ml)	15.0 ± 1.27	15.6 ± 1.44
QUICKI	0.32	0.32
Type 2 diabetes		
Glycemia >7 mmol/l or treatment	12/55 (22)	9/39 (23)
Lipids homeostasis		
Cholesterol (mmol/l)	5.32 ± 0.12	5.37 ± 0.14
HDL cholesterol (mmol/l)	1.34 ± 0.06	1.33 ± 0.05
Triglycerides (mmol/l)	1.49 ± 0.11	1.47 ± 0.14
Adipokines		
Leptin (ng/ml)	62.4 ± 3.80	61.2 ± 4.13
Adiponectin (μg/ml)	7.01 ± 0.42	6.81 ± 0.51
Risk factors		
HDL <1.03 mmol/l (M), <1.29 mmol/l (F)	25 (45)	18 (46)
Hypertension ≥130/85 mmHg	24 (44)	16 (41)
Glucose ≥5.6 mmol/l	25 (45)	21 (54)
Triglycerides ≥1.7 mmol/l	17 (31)	11 (28)
Inflammatory factors		
TNF-α (pg/ml)	1.79 ± 0.07	1.87 ± 0.09
Interleukin 6 (pg/ml)	2.63 ± 0.18	2.74 ± 0.23
High-sensitivity CRP (mg/dl)	0.89 ± 0.09	0.91 ± 0.12
Orosomucoid (g/l)	0.99 ± 0.03	0.99 ± 0.03
Serum amyloid A (μg/ml)	31.5 ± 7.6	35.1 ± 10.1
Hepatic factors		
AST (IU/l)	22.8 ± 1.57	23.7 ± 1.98
Alanine aminotransferase (IU/l)	32.8 ± 3.76	33.5 ± 4.40
γGT (mg/dl)	36.8 ± 2.95	37.1 ± 3.31

Data are the means ± SE or *n* (%). Histology of hepatic biopsies was suitable for 39 individuals of the cohort, designated as the "liver subset." No significant differences were observed between the two groups.

(13). To our knowledge, no correlation between WAT macrophages and clinical or metabolic parameters of insulin resistance, type 2 diabetes, or other obesity-associated diseases has been reported in human studies.

In humans, subcutaneous WAT is easier to obtain than visceral WAT for biopsies, and it is not known whether macrophage infiltration varies among WAT depots. A recent study indicates that visceral WAT contains increasing amounts of macrophages in obese individuals, similar to that observed in subcutaneous WAT (C. Curat, A. Bouloumié, personal communication). It is well known that visceral fat mass accumulation is linked to obesity comorbidities and is associated with increased risk of cardiovascular and hepatic diseases (14–17). A wide range of liver lesions known as nonalcoholic fatty liver disease (NAFLD) has been shown to be associated with intra-abdominal fat mass in both men and women, independent of subcutaneous fat levels (18). NAFLD includes a large spectrum of lesions, from simple steatosis, considered to be benign, to inflammatory lesions called nonalcoholic steatohepatitis, which have the potential to progress to fibrosis, cirrhosis, and hepatocellular carcinoma. Omental WAT is drained by the portal venous system and has direct circulating connection with the liver. Thus, deregulated adipose production of fatty acids, adipokines, and/or inflammatory factors by omental WAT in obesity might have an impact on liver function and morphology, as suggested by the "portal hypothesis" (19,20). However, there are no studies describing a potential relationship between infiltration of macrophages in omental WAT and the complications of obesity, in particular liver diseases.

We tested the hypothesis that increased recruitment of macrophages in omental WAT is one of the cellular mechanisms linking increased visceral adiposity to obesity-induced biological and histopathological liver abnormalities. We evaluated the degree of macrophage infiltration in omental WAT compared with subcutaneous WAT in a cohort of 55 morbidly obese patients, and we examined potential relationships with clinical, metabolic, and hepatic anomalies.

RESEARCH DESIGN AND METHODS

A total of 55 morbidly obese subjects involved in a gastric surgery program were recruited at the Department of Nutrition of the Hôtel-Dieu Hospital (Paris, France). Clinical and biological parameters are shown in Table 1. Preoperative evaluation included medical history, physical, nutritional, cardiopulmonary, and psychological assessments. Obese subjects had been weight stable for at least 3 months before surgery. All patients met the criteria for obesity surgery, i.e., BMI ≥40 or ≥35 kg/m^2 with at least two significant comorbidities (hypertension, type 2 diabetes, or dyslipidemia). They were excluded if they had evidence of acute or chronic inflammatory disease, infectious diseases, cancer, and/or known alcohol consumption (>20 g per day), as well as other causes of liver diseases (viral hepatitis, hemochromatosis, Wilson's disease, autoimmune hepatitis, or α-1 antitrypsine deficit). The ethics committees of the Hôtel-Dieu Hospital approved the clinical investigations, and all subjects gave informed consent. Increased metabolic risk factors were present in 31–45% of the whole population. The risk factors presented in Table 1 are surrogates of the metabolic syndrome components, as defined by

the AHA (American Heart Association) science advisory and coordinating committee and by the NHLBI (National Heart, Lung, and Blood Institute). According to the criteria of fasting glycemia >7 mmol/l or treatment, 12 of 55 subjects were type 2 diabetic patients (Table 1). Among them, seven were treated with metformin and/or hypolipemic drugs.

Immunomorphologic analysis of adipose tissue. Paired omental WAT and subcutaneous WAT biopsies were obtained during gastric surgery. A portion of each WAT biopsy was immediately transferred into liquid nitrogen before RNA analysis. The other part was fixed overnight at 4°C in 4% paraformaldehyde and then processed for standard paraffin embedding. Sections of 5 μm were stained as described below and observed under a Zeiss 20 Axiostar Plus microscope (Zeiss, Göttingen, Germany). Digital images were captured by a camera (triCCD; Sony, Paris, France). Adipocyte diameters were measured, using PerfectImage software (Claravision, Orsay, France). Immunohistochemical detection of HAM56 (Dako Cytomation, Trappes, France) was performed with the avidin-biotin peroxidase method (21). Dewaxed sections were processed through the following incubation steps: *1*) antigen unmasking by 750 W microwave washing in a solution of 10 mmol/l citrate buffer, pH 6.0, three times; *2*) 3% hydrogen peroxide in water for 15 min to block endogenous peroxidases; *3*) Tris-buffered saline/Tween 20/casein 0.02 mol/l solution (TBS-TC) for 10 min; *4*) monoclonal mouse antibodies diluted 1:100 (1 h) in TBS-TC at 4°C; and *5*) multilink anti-mouse biotinylated immunoglobulins (Dako Cytomation) diluted 1:200 in TBS-TC for 20 min. Standard streptavidin-biotin-peroxidase complex method was applied using a commercially available kit (AbCYS Biospa, Milan, Italy), and the staining was visualized using diaminobenzidine (Dako Cytomation). Slides were counterstained with Mayer's hematoxylin. Method specificity tests were performed by omission of primary antibodies and use of preimmune serum. Adipocytes and HAM56+ cells were counted in 10 different randomly chosen areas in each processed slide with 40× magnification. Counting was performed blindly by three independent observers.

Liver histopathology. A liver biopsy was performed in each of the 55 enrolled patients, and a subset of 39 biopsies of this cohort was suitable for histological evaluation. Liver samples were fixed in formalin, embedded in paraffin, and routinely stained (hematoxylin and eosin, Masson's trichrome, picrosirius red, and Perls' staining). Slides were coded and analyzed by a single expert pathologist blind to the identity of the biopsy. Major histopathological features were recorded and semiquantitated according to previously published criteria (22). The amount of steatosis was scored as followed: 0 (0–5%), 1 (5–33%), 2 (34–66%), and 3 (>67%). Foci of lobular inflammation were defined as two or more inflammatory cells averaged from three to four fields counted at 20× magnification and then classified as 0 (no foci), 1 (<2), 2 (2–4), and 3 (>4). Portal inflammation was evaluated by inflammatory infiltrate, mainly mononuclear cells, assessed from low magnification and scored 0 (none to minimal) or 1 (greater than minimal). Fibrosis was classed as stage 0 (none), stage 1 (1a-b zone 3 perisinusoidal fibrosis only, 1c portal fibrosis only), stage 2 (zone 3 perisinusoidal fibrosis and periportal fibrosis without bridging), stage 3 (bridging fibrosis), and stage 4 (cirrhosis, probable and definite). Because the stage of fibrosis and the grade of inflammation are part of the spectrum that differentiates simple steatosis from steatohepatitis, we established an overall score of severity by adding elementary scores of fibrosis, portal inflammation, and lobular inflammation. Using this method, histopathological liver damage was either absent (score = 0), mild (1 or 2), or marked (>2).

Laboratory tests. Blood samples were collected after an overnight fast of 12 h. Plasma glucose, cholesterol, and HDL cholesterol levels were measured enzymatically. Serum insulin concentrations were determined with an immunoradiometric assay kit (Bi-Insulin IRMA; CisBio International, Saclay, France). Serum amyloid A concentrations were measured, using an enzyme-linked immunosorbent assay Cytoscreen immunoassay kit (BioSource International, Camarilla, CA). Serum leptin and adiponectin were determined, using radioimmunoassay kits from Linco Research (St. Louis, MO), according to the manufacturer's instructions. Interleukin 6 and tumor necrosis factor (TNF)-α serum levels were measured by an ultrasensitive enzyme-linked immunosorbent assay system (Quantikine; R&D System Europe, Abingdon, U.K.). Orosomucoid and high-sensitivity C-reactive protein were measured using an Immage automatic immunoassay system (Beckman-Coulter, Fullerton, CA). Insulin sensitivity was evaluated by the quantitative insulin sensitivity check index (QUICKI) method. Calculation was performed from fasting glucose and insulin as previously described (23).

Statistical analyses. Data are the means ± SE. The Shapiro-Wilcoxon test was used to test the Gaussian distribution of all clinical and biological parameters. Skewed variables were log-transformed to normalize their distribution before statistical analyses. We verified the normality of log-transformed variables. Relationships between WAT macrophage infiltration and quantitative clinical and biological variables were explored by logistic regression. ANOVA was used for quantitative traits and χ^2 test for noncontinuous values. We used a stepwise variable method of selection to predict the degree of WAT macrophage infiltration and the score of liver lesions. Statistical analysis was performed with JMP statistics software (SAS Institute, Cary, NC). $P < 0.05$ was considered significant.

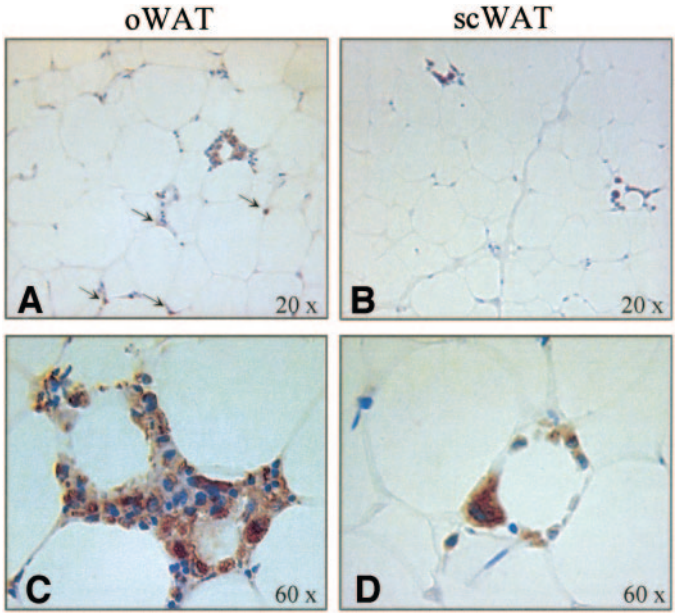

FIG. 1. Immunohistochemical detection of HAM 56+ macrophages in omental WAT (*A* and *C*) and subcutaneous WAT (*B* and *D*) of one representative obese woman. *A*: Macrophages are both dispersed into the omental WAT parenchyma and in crown arrangement (*A*, arrow heads). *B*: Only crown-like structures are visible in subcutaneous WAT parenchyma. Higher magnification (60×) of crown structures in omental WAT (*C*) and subcutaneous WAT (*D*). oWAT, omental WAT; scWAT, subcutaneous WAT.

RESULTS

Macrophages were identified on immunoreactivity for the HAM56 marker in paired subcutaneous WAT and omental WAT biopsies sampled from the same obese individual. Numerous single HAM56+ cells were dispersed throughout the parenchyma in omental WAT (Fig. 1*A*), whereas these isolated immune cells were virtually absent in subcutaneous WAT (Fig. 1*B*). A "crown" organization of macrophages around a single adipocyte was observed in omental WAT (Fig. 1*A* and *B*), with two adjacent crowns with numerous macrophages sometimes visible (Fig. 1*C*). By contrast, in the crowns present in subcutaneous WAT, there were markedly fewer macrophages (Fig. 1*D*).

To quantify the abundance of macrophage infiltrates, HAM56+ cells were systematically counted on each processed slide. The number of macrophages was normalized to 100 adipocytes for comparison between tissues and between patients. The number of infiltrated macrophages was twice as high in omental WAT as in subcutaneous WAT (30.1 ± 2.4 vs. 14.8 ± 1.8%, respectively; $P < 0.0001$) (Fig. 2*A*). Higher expression (by RT–quantitative PCR) of two macrophage markers (CD11b and CD87) in omental than in subcutaneous WAT confirmed direct cell quantification (data not shown).

Because adipocyte hypertrophy is a factor determining WAT macrophage infiltration (3,6), we assessed the relationship between adipose cell diameter and the percentage of macrophages in omental and subcutaneous WAT. The mean diameter of omental adipose cells was significantly smaller than that of subcutaneous cells (73.1 ± 1.8 vs. 86.3 ± 1.7 μm, $P < 0.0001$) (Fig. 2*B*). Thus, there was

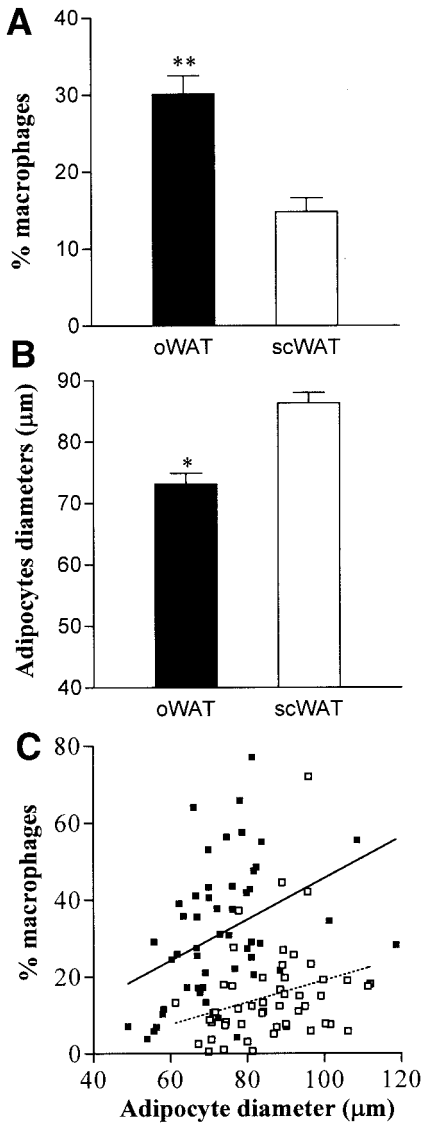

FIG. 2. Percentage of macrophage infiltration (*A*) and adipocyte diameter (*B*) in omental WAT (■) and subcutaneous WAT (□). Data are the means ± SE. *$P < 0.01$; **$P < 0.001$. *C*: Correlations between the percentage of macrophage infiltration and adipocyte diameter in omental WAT (■) and subcutaneous WAT (□). Lines are linear regressions in omental WAT (solid line) and in subcutaneous WAT (dotted line). oWAT, omental WAT; scWAT, subcutaneous WAT.

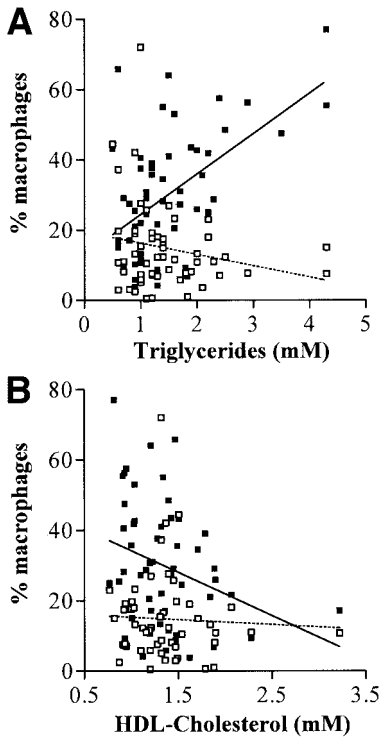

FIG. 3. Correlations between the percentage of macrophages and triglycerides (*A*) or HDL cholesterol (*B*) in omental WAT (■) and subcutaneous WAT (□). Lines are linear regressions in omental WAT (solid line) and in subcutaneous WAT (dotted line).

greater macrophage infiltration in omental than in subcutaneous WAT, despite the smaller adipose cell size. Within each fat depot, there was a positive correlation between adipose cell diameter and the number of infiltrating macrophages ($R^2 = 0.19$, $P = 0.001$, in omental WAT, and $R^2 = 0.13$, $P = 0.010$, in subcutaneous WAT) (Fig. 2*C*). Thus, fat cell hypertrophy is not the sole determinant of macrophage recruitment into WAT; it is also dependent on the localization of anatomic fat depot. Systemic metabolic or hormonal factors cannot explain this site-associated difference because both types of WAT were obtained from the same subject. There was no significant correlation between the degree of macrophage infiltration in omental WAT and that in subcutaneous WAT in the same individual ($R^2 = 0.08$, $P = 0.58$).

Linear regression was performed to examine the relationships between the percentage of macrophage infiltration in omental WAT and clinical and biological parameters. The percentage of macrophages in omental WAT was significantly related to age at surgery ($R^2 = 0.07$, $P = 0.045$) in the whole population of 55 subjects. Thus, subsequent linear regressions were adjusted for age. In contrast, only a trend toward a correlation ($P = 0.06$) was found with the subject's BMI. We found that omental WAT macrophage infiltration was positively related to fasting concentrations of glucose ($R^2 = 0.17$, $P = 0.02$) and insulin ($R^2 = 0.17$, $P = 0.02$) and negatively associated with a marker of insulin sensitivity, QUICKI ($R^2 = 0.15$, $P = 0.04$). Strong positive and negative relationships were found with triglycerides ($R^2 = 0.25$, $P = 0.0009$) (Fig. 3*A*) and with HDL cholesterol ($R^2 = 0.21$, $P = 0.005$), respectively (Fig. 3*B*). The hepatic parameters aspartate aminotransferase (AST; $R^2 = 0.19$, $P = 0.009$) and γ-glutamyltranspeptidase (γGT; $R^2 = 0.15$, $P = 0.04$) were also associated with the percentage of macrophages in omental WAT. Strikingly, none of these relationships was found with the amount of macrophage infiltration in subcutaneous WAT (Fig. 3). We found no association between omental WAT macrophage infiltration and systemic inflammatory parameters (orosomucoid, serum amyloid A, or high-sensitivity C-reactive protein) and a trend toward an association for interleukin 6 ($R^2 = 0.13$, $P = 0.09$) and TNF-α ($R^2 = 0.14$, $P = 0.07$). ANOVA revealed that the degree of macrophage infiltration in omental WAT was also strongly associated with sex ($P = 0.004$) as well as with triglycerides >1.7 mmol/l ($P = 0.01$) and more moderately with HDL cholesterol–linked metabolic risk ($P = 0.09$). After adjustment of all significant quantitative variables for age and sex, only fasting triglyceride concentrations ($P = 0.026$) and the triglyceride–to–HDL cholesterol ratio ($P = 0.012$) remained associated with the percentage of macrophages in omental WAT, indicating a substantial sex and age effect on other vari-

TABLE 2
Clinical and biological parameters of 39 obese patients classified by the severity of liver fibroinflammatory scores

	Hepatic fibroinflammatory score			
	Absent (0)	Mild (1–2)	Marked (>2)	*P*
n	7	17	15	—
F/M	6/1 (86)	16/1 (94)	9/6 (60)	—
Age (years)	41.4 ± 4.6	37.4 ± 2.5	39.0 ± 2.5	0.67
BMI (kg/m^2)	44.5 ± 3.5	47.6 ± 1.5	49.8 ± 1.9	0.28
Glucose (mmol/l)	5.57 ± 0.39	5.79 ± 0.37	7.17 ± 0.71*	0.11
Insulin (μU/ml)	8.9 ± 1.75	14.5 ± 1.76	19.3 ± 2.82†	0.036
QUICKI	0.35	0.32‡	0.30*	0.009
Type 2 diabetes	0	3 (18)	6 (40)	0.09
Cholesterol (mmol/l)	5.63 ± 0.24	5.23 ± 0.18	5.47 ± 0.29	0.56
HDL cholesterol (mmol/l)	1.53 ± 0.15	1.35 ± 0.09	1.24 ± 0.06	0.26
Triglycerides (mmol/l)	1.17 ± 0.10	1.26 ± 0.17	1.89 ± 0.29*	0.11
Triglyceride–to–HDL cholesterol ratio	0.84 ± 0.40	1.04 ± 0.25	1.74 ± 0.27	0.10
Leptin (ng/ml)	49.8 ± 5.52	69.4 ± 6.00	56.7 ± 7.9	0.14
Adiponectin (μg/ml)	7.19 ± 1.35	7.82 ± 0.90	5.59 ± 0.59*	0.11
TNF-α (pg/ml)	1.87 ± 0.16	1.85 ± 0.18	1.90 ± 0.11	0.84
Interleukin 6 (pg/ml)	2.34 ± 0.61	2.54 ± 0.28	3.12 ± 0.41	0.33
High-sensitivity CRP (mg/dl)	1.09 ± 0.41	0.79 ± 0.13	0.97 ± 0.22	0.82
Orosomucoid (g/l)	1.00 ± 0.07	1.04 ± 0.05	0.93 ± 0.04	0.37
Serum amyloid A (μg/ml)	21.8 ± 8.6	36.8 ± 15.5	39.4 ± 19.7	0.83
HDL <1.03 mmol/l (M), <1.29 mmol/l (F)	2 (29)	8 (44)	8 (53)	0.55
Hypertension ≥130/85 mmHg	2 (29)	6 (35)	8 (53)	0.44
Glucose ≥5.6 mmol/l	3 (43)	8 (44)	10 (66)	0.43
Triglycerides ≥1.7 mmol/l	0	3 (18)‡	8 (53)*	0.015
Macrophages in omental WAT (%)	22.2 ± 7.2	24.9 ± 3.8‡	40.7 ± 4.5*	0.016
Adipocyte diameter omental WAT (μm)	72.8 ± 7.6	72.3 ± 4.2	75.2 ± 3.1	0.75
Macrophages in subcutaneous WAT (%)	15.5 ± 1.53	14.9 ± 2.47	12.2 ± 3.13	0.12
Adipocyte diameter in subcutaneous WAT (μm)	95.8 ± 5.05	84.0 ± 3.6	85.2 ± 2.8	0.11
AST (IU/l)	19.7 ± 3.3	19.6 ± 1.2	30.4 ± 4.3*	0.012
Alanine aminotransferase (IU/l)	27.7 ± 10.9	25.9 ± 2.8	45.3 ± 9.3†	0.043
γGT (mg/dl)	32.3 ± 10.9	32.6 ± 3.9	45.4 ± 5.3†	0.045

Data are the means ± SE, R^2, or *n* (%). *P* values were obtained by ANOVA test for continuous values or by χ^2 test for noncontinuous values. A different label (*, †, ‡) indicates significant differences using Student's *t* test for each pair ($P < 0.05$ for *mild vs. marked, †absent vs. marked, and ‡absent vs. mild, respectively).

ables. In addition, the association between triglycerides and omental WAT macrophages remained statistically significant after adjustment for diabetes status ($P = 0.03$) or exclusion of patients undergoing hypolipemic treatment ($P = 0.04$).

We used a forward-variable method of selection to identify the best model to estimate the degree of macrophage infiltration. Variables were added according to a criterion based on the decrease of the error sum of squares (criterion based on *F* value of the ANOVA). This model showed that macrophage infiltration in omental WAT was best predicted by fasting triglyceride values, sex, and age. These parameters can be used to derive an equation that estimates the percentage of macrophage infiltration in omental WAT as follows: 12.163 + 9.98 (× 1 if sex = M, or × 0 if sex = F) + 0.332 × age + 11.491 × (log fasting triglyceride in mmol/l). This model explains 30% of the variability of macrophage infiltration in omental WAT.

To test the hypothesis that increased macrophage infiltration in omental WAT could be involved in the severity of liver lesions, we performed a detailed semiquantitative histological analysis of liver biopsies in 39 subjects. Clinical and biological parameters were not significantly different between this group and the entire studied population (Table 1). We used a score of histological severity that takes into consideration both inflammatory parameters (lobular and portal inflammation) and fibrosis. This score identified three groups of patients according to the severity of hepatic lesions (absent, mild, and marked) (Table 2). In our population of 39 obese subjects, 17.9% had no histological severity and 43.5% presented mild inflammatory lesions (score 1 to 2), whereas 38.5% had marked lesions (score >2). Patients with marked lesions had a more severe biological phenotype. They had significantly increased insulinemia, reduced QUICKI, and higher hepatic factors (AST, alanine aminotransferase, and γGT) compared with the mild and/or absent groups (Table 2). The proportion of type 2 diabetic patients in this group was higher, although not significantly ($P = 0.09$), than in the group with mild hepatic lesions (Table 2). There were trends toward association between the severity of hepatic lesions and increased fasting glucose, increased fasting triglycerides, and decreased adiponectin plasma concentrations, which can be explained by significant differences between patients with mild and marked lesions.

In agreement with our initial hypothesis, omental WAT macrophage accumulation was significantly associated with severity of fibroinflammatory liver damage ($P = 0.016$) (Table 2). This effect was also observed when the subgroup ($n = 31$) of women was analyzed separately ($P = 0.030$) (data not shown). The percentage of macrophage infiltration in omental WAT was almost double in the group with marked fibroinflammatory lesions compared with the group without or with mild alterations (Table 2 and Fig. 4*A*). By contrast, the score of steatosis alone was not significantly related to macrophage infiltra-

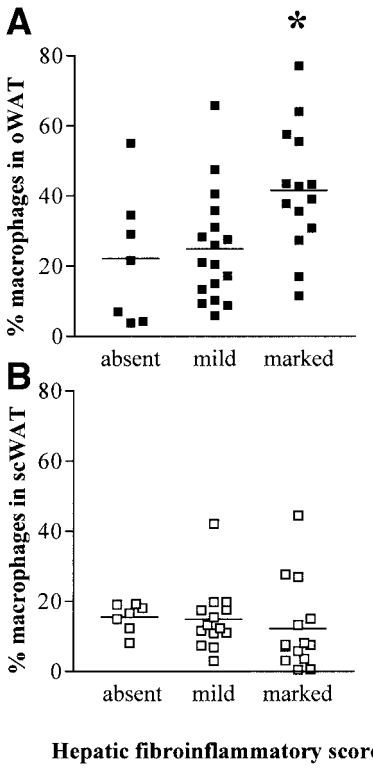

FIG. 4. Percentage of macrophages in omental WAT (*A*) and in subcutaneous WAT (*B*) in 39 subjects scored for hepatic fibroinflammatory lesions. Horizontal lines are the mean value in each group. *$P < 0.05$ using Student's test for each pair, compared with mild and absent group. oWAT, omental WAT; scWAT, subcutaneous WAT.

tion in omental WAT (data not shown). No correlation was found between subcutaneous WAT macrophage infiltration and the severity of hepatic lesions ($P = 0.12$) (Table 2 and Fig. 4*B*). Using a stepwise selection algorithm based on Akaike information criteria, we fit the best logistic regression, using significant variables to predict the severity of hepatic lesions (marked group). The best predictive model included the three variables: adiponectinemia, AST levels, and percentage of macrophage in omental WAT (total deviance 50 with 36 degrees of freedom [df], residual deviance 35 with 33 df, Akaike information criteria = 42.923). The predicting odds ratio for being in the severe class of hepatic fibroinflammatory lesions can be computed as: $e(-5.217 + 2.103 \times \log \text{AST} + 0.060 \times \%$ macrophages in omental WAT $- 1.797 \times \log$ adiponectin). Interestingly, the replacement of measured omental WAT macrophages by values calculated with the equation defined above using age, sex, and triglycerides did not significantly alter this predictive equation.

DISCUSSION

The presence of resident macrophages in WAT and their increased recruitment during obesity was recently established in both humans and rodents (3–6). At present, the pathological consequences of macrophage infiltration in WAT remain hypothetical. One key unanswered question is whether increased macrophage infiltration in a particular WAT depot is associated with specific obesity comorbidities. This study clearly shows that macrophage accumulation in WAT is dependent on anatomical localization. Indeed, there is twice as much infiltration in omental as in subcutaneous WAT. In addition, omental WAT macrophage accumulation is significantly associated with severe hepatic fibroinflammatory lesions in morbid obesity in humans.

To our knowledge, this is the first study to demonstrate a difference between macrophage accumulation in omental and subcutaneous WAT, based on direct quantification of infiltrating HAM56+ cells on adipose tissue slides. CD68+ cells have been shown to be more common in visceral than in subcutaneous WAT in normal-weight human subjects (10), suggesting that increased omental WAT infiltration includes nonobese individuals. The degree of macrophage infiltration represents a new WAT site–related difference, in addition to distinct metabolic capacities, gene expression, secretory functions, and hormonal responsiveness (24). The actual factors responsible for the higher recruitment of macrophages in omental than in subcutaneous WAT are unknown. In animal models of obesity, a relationship has been shown between adipose cell size and the amount of macrophages in the stroma vascular fraction of WAT (3). The current study indicates that in addition to adipose cell hypertrophy, depot-specific vascularization and/or innervation might explain distinct WAT infiltration with immune cells.

Our most important finding is that omental WAT macrophage accumulation is significantly associated with severe hepatic fibroinflammatory lesions, which were found in ~40% of the morbidly obese population. This deleterious association appears to be specific for macrophage infiltration in omental WAT because no relationship was observed between histological liver damage and the amount of macrophages in subcutaneous WAT. The robust correlation found in the entire population between omental WAT macrophages and two hepatic parameters, AST and γGT, supports the hypothesis that omental WAT macrophage infiltration contributes to worsen liver damage. This is the first report identifying an association between macrophage infiltration in WAT and a comorbidity in human obesity.

The molecular mechanisms linking macrophage infiltration in omental WAT and hepatic alterations are yet to be investigated. Recent studies in humans have evaluated the implication of adipokines, such as leptin, adiponectin, and TNF-α, in the pathogenesis of liver diseases (25–27). In our population of massively obese patients, neither leptin nor TNF-α circulating levels were significantly associated with the severity of hepatic lesions. Regarding adiponectin, previous studies (26,27) have shown that low circulating levels associate with worsening grades of hepatic necroinflammation. In the current study, the obese patients with marked hepatic fibroinflammatory lesions displayed significantly reduced adiponectin levels. These observations suggest a role for adiponectin to protect from liver inflammatory damage. Whether macrophage infiltration in omental WAT contributes to reduce adiponectin production in human obesity is currently unknown.

In our correlation study, triglycerides were the best predictor of omental WAT macrophage infiltration and increased with the severity of hepatic lesions. Because triglycerides are also increased in type 2 diabetes, we examined various parameters in diabetic versus nondiabetic subjects in our population. Our data suggest that diabetes is not a major determinant of omental WAT macrophage accumulation. Regarding the severity of hepatic lesions, there is a trend toward an increased number of diabetic subjects in the "marked" groups versus the "mild" group. It should be noted, however, that the small

number of diabetic subjects in our cohort might account for the absence of statistical differences. Further studies including obese and nonobese diabetic subjects are needed to assess whether diabetes contributes to aggravate liver lesions in humans.

NAFLD includes a wide spectrum of diseases, from pure steatosis to nonalcoholic steatohepatitis in the absence of significant alcohol consumption. Although steatosis is considered a nonprogressive disease, nonalcoholic steatohepatitis may deteriorate in advanced chronic liver diseases, cirrhosis, and hepatocellular carcinoma (28). Worsening of steatosis is associated with the progression of fibrosis in patients with chronic hepatitis (29,30). The histopathological criteria that differentiate steatosis from nonalcoholic steatohepatitis have not been uniformly defined. Fibrosis is an obvious marker of severity, and a score has recently been proposed (22). In addition, portal or lobular inflammation tends to result in the development of fibrosis. Therefore, in the current study, we scored the severity of liver histopathology with a semiquantitative evaluation of inflammation and fibrosis, two patterns of which are part of nonalcoholic steatohepatitis in most published reports. This classification identified a group of patients with severe fibroinflammation and a high degree of macrophage infiltration in omental WAT. Current understanding of the causes, mechanisms, and implications of obesity-associated liver damage is poor. Recent stable isotope studies in obese NAFLD patients demonstrated that nearly 60% of the triacylglycerol found in the liver arose from the nonesterified fatty acid pool in the serum, 60–80% of which comes from adipose tissue lipolysis (31). Activated macrophages secrete numerous cytokines that activate lipolysis through various signaling pathways converging on phosphorylation of hormone-sensitive lipase or its associated proteins, such as perilipins (32). In obese individuals, the presence of infiltrating macrophages in omental WAT might increase nonesterified fatty acid delivery to the liver and aggravate steatosis, paving the way for more severe hepatic damage. As macrophage infiltration increases in omental WAT, inflammatory molecules could reach the liver directly through the portal system and act in concert with locally produced proinflammatory factors (33) to increase liver cell apoptosis, a prerequisite for developing fibrosis. To what extent this deleterious process is triggered by factors released from omental WAT macrophages or from resident macrophages in liver (Küppfer cells) remains to be determined. Although measurement of inflammatory molecules in the portal system would be informative, this is not feasible in humans.

A recent longitudinal study in NAFLD patients, using sequential liver biopsy, showed that fibrosis progresses in 37% of patients, with a high variability in the rate of progression among subjects (34). This emphasizes the need for predictive parameters to evaluate the amount of hepatic damage. A clinical-biological score combining BMI, age, alanine aminotransferase, and triglycerides has been proposed to predict septal fibrosis in overweight patients (35). In this study, we used a stepwise approach to find the best clinical and biological predictors of severe hepatic fibroinflammatory lesions from data, including the measurement of candidate inflammatory biomolecules and the amount of macrophages in omental WAT. A model combining adiponectin, AST, and omental WAT macrophages (directly measured or estimated from age, sex, and triglycerides) was shown to be the best combination associated with the degree of fibroinflammatory hepatic lesions. This finding supports a deleterious role for macrophage infiltration in omental WAT and reduced circulating adiponectin levels in the worsening of hepatic fibroinflammatory lesions.

In conclusion, this is the first demonstration of a link between omental WAT macrophage accumulation and severe hepatic inflammatory damages in human obesity. The predictive equation that we propose could be used as a surrogate for liver biopsy in morbidly obese individuals to estimate the severity of hepatic damage. Currently, the validity of this model has to be considered only for morbidly obese patients. Future investigations of independent human cohorts with different degrees of liver disease and BMI should validate its accuracy and potential general application. In addition, because we found significant links among macrophage recruitment, triglycerides, and HDL cholesterol, the consequences of omental WAT macrophage infiltration on cardiovascular disease related to obesity could be of interest in future studies.

ACKNOWLEDGMENTS

This work was supported by INSERM "Avenir contract," the Programme Hospitalier de Recherche Clinique of Assistance Publique Hôpitaux de Paris (AOR0276), the Alfediam Association (Clinical Research Contract 2002/2003), and a grant from ANR (French National Agency of Research (program no. ANR05-PCOD-030-02). R.C. was funded by INSERM, Agence Déléguée Régionale Paris 6, Saint-Antoine, Paris, and the Conseil Régional de l'Ile-de-France and received a grant from the Association Française d'Etude et de Recherche sur l'Obésité/Roche Association (France).

Florence Marchelli contributed to the database constitution. The authors thank Roche Pharma France (Gérard Babany and Dale Roche) for editing the manuscript.

REFERENCES

1. Gordon S: The macrophage. *Bioessays* 17:977–986, 1995
2. Loftus TM, Kuhajda FP, Lane MD: Insulin depletion leads to adipose-specific cell death in obese but not lean mice. *Proc Natl Acad Sci U S A* 95:14168–14172, 1998
3. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808, 2003
4. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830, 2003
5. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A: From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 53:1285–1292, 2004
6. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, Bouillot JL, Bouloumie A, Barbatelli G, Cinti S, Svensson PA, Barsh GS, Zucker JD, Basdevant A, Langin D, Clement K: Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* 54:2277–2286, 2005
7. Cousin B, Munoz O, Andre M, Fontanilles AM, Dani C, Cousin JL, Laharrague P, Casteilla L, Penicaud L: A role for preadipocytes as macrophage-like cells. *FASEB J* 13:305–312, 1999
8. Charriere G, Cousin B, Arnaud E, Andre M, Bacou F, Penicaud L, Casteilla L: Preadipocyte conversion to macrophage: evidence of plasticity. *J Biol Chem* 278:9850–9855, 2003
9. Imai T, Takakuwa R, Marchand S, Dentz E, Bornert JM, Messaddeq N, Wendling O, Mark M, Desvergne B, Wahli W, Chambon P, Metzger D: Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. *Proc Natl Acad Sci U S A* 101:4543–4547, 2004
10. Bornstein SR, Abu-Asab M, Glasow A, Path G, Hauner H, Tsokos M,

Chrousos GP, Scherbaum WA: Immunohistochemical and ultrastructural localization of leptin and leptin receptor in human white adipose tissue and differentiating human adipose cells in primary culture. *Diabetes* 49:532–538, 2000
11. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS: Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 46:2347–2355, 2005
12. Clement K, Viguerie N, Poitou C, Carette C, Pelloux V, Curat CA, Sicard A, Rome S, Benis A, Zucker JD, Vidal H, Laville M, Barsh GS, Basdevant A, Stich V, Cancello R, Langin D: Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *FASEB J* 18:1657–1669, 2004
13. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW: CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 116:115–124, 2006
14. Haslam DW, James WP: Obesity. *Lancet* 366:1197–1209, 2005
15. Shankar SS, Steinberg HO: Obesity and endothelial dysfunction. *Semin Vasc Med* 5:56–64, 2005
16. Qian Y, Fan JG: Obesity, fatty liver and liver cancer. *Hepatobiliary Pancreat Dis Int* 4:173–177, 2005
17. Diehl AM: Hepatic complications of obesity. *Gastroenterol Clin North Am* 34:45–61, 2005
18. Westerbacka J, Corner A, Tiikkainen M, Tamminen M, Vehkavaara S, Hakkinen AM, Fredriksson J, Yki-Jarvinen H: Women and men have similar amounts of liver and intra-abdominal fat, despite more subcutaneous fat in women: implications for sex differences in markers of cardiovascular risk. *Diabetologia* 47:1360–1369, 2004
19. Frayn KN: Lipoproteins in health and disease. *Eur J Clin Nutr* 54: 273, 2000
20. Frayn KN: Visceral fat and insulin resistance: causative or correlative? *Br J Nutr* 83 (Suppl. 1):71–77, 2000
21. Hsu SM, Raine L: Protein A, avidin, and biotin in immunohistochemistry. *J Histochem Cytochem* 29:1349–1353, 1981
22. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ: Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 41:1313–1321, 2005
23. Katz LE, DeLeon DD, Zhao H, Jawad AF: Free and total insulin-like growth factor (IGF)-I levels decline during fasting: relationships with insulin and IGF-binding protein-1. *J Clin Endocrinol Metab* 87:2978–2983, 2002
24. Lafontan M, Berlan M: Do regional differences in adipocyte biology provide new pathophysiological insights? *Trends Pharmacol Sci* 24:276–283, 2003
25. Marra F, Aleffi S, Bertolani C, Petrai I, Vizzutti F: Adipokines and liver fibrosis. *Eur Rev Med Pharmacol Sci* 9:279–284, 2005
26. Hui JM, Hodge A, Farrell GC, Kench JG, Kriketos A, George J: Beyond insulin resistance in NASH: TNF-alpha or adiponectin? *Hepatology* 40:46–54, 2004
27. Musso G, Gambino R, Biroli G, Carello M, Faga E, Pacini G, De Michieli F, Cassader M, Durazzo M, Rizzetto M, Pagano G: Hypoadiponectinemia predicts the severity of hepatic fibrosis and pancreatic beta-cell dysfunction in nondiabetic nonobese patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 100:2438–2446, 2005
28. Schaffler A, Scholmerich J, Buchler C: Mechanisms of disease: adipocytokines and visceral adipose tissue–emerging role in nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2:273–280, 2005
29. Castera L, Hezode C, Roudot-Thoraval F, Bastie A, Zafrani ES, Pawlotsky JM, Dhumeaux D: Worsening of steatosis is an independent factor of fibrosis progression in untreated patients with chronic hepatitis C and paired liver biopsies. *Gut* 52:288–292, 2003
30. Fartoux L, Chazouilleres O, Wendum D, Poupon R, Serfaty L: Impact of steatosis on progression of fibrosis in patients with mild hepatitis C. *Hepatology* 41:82–87, 2005
31. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ: Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 115:1343–1351, 2005
32. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C: Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J Lipid Res* 45:1169–1196, 2004
33. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE: Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 11:183–190, 2005
34. Adams LA, Sanderson S, Lindor KD, Angulo P: The histological course of nonalcoholic fatty liver disease: a longitudinal study of 103 patients with sequential liver biopsies. *J Hepatol* 42:132–138, 2005
35. Ratziu V, Giral P, Charlotte F, Bruckert E, Thibault V, Theodorou I, Khalil L, Turpin G, Opolon P, Poynard T: Liver fibrosis in overweight patients. *Gastroenterology* 118:1117–1123, 2000