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Visceral fat accumulation is an indicator of adipose tissue macrophage infiltration in women

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ABSTRACT

We tested the hypothesis that visceral obesity is the best correlate of abdominal adipose tissue macrophage infiltration in women. Omental and subcutaneous fat samples were surgically obtained from 40 women (age, 47.0 ± 4.0 years; body mass index, 28.4 ± 5.8 kg/m^2). CD68+ cells were identified using fluorescence immunohistochemistry. Expression of macrophage markers was measured by real-time reverse transcriptase polymerase chain reaction. Body composition and fat distribution were measured by dual-energy x-ray absorptiometry and computed tomography, respectively. Mean CD68+ cell percentage tended to be higher in subcutaneous (18.3%) compared with omental adipose tissue (15.5%, $P = .07$). Positive correlations were observed between CD68+ cell percentage as well as CD68 messenger RNA expression in a given depot vs the other ($P \leq .01$). Visceral adipose tissue area and omental adipocyte diameter were positively related to CD68+ cell percentage in omental fat ($r = 0.52$ and $r = 0.35$, $P \leq .05$). Total and visceral adipose tissue areas as well as subcutaneous adipocyte diameter were significantly correlated with CD68+ cell percentage in subcutaneous adipose tissue ($0.32 \leq r \leq 0.40$, $P \leq .05$). Adipose tissue areas and subcutaneous adipocyte diameter were also significantly associated with expression of commonly used macrophage markers including CD68 in the subcutaneous fat compartment ($0.32 \leq r \leq 0.57$, $P \leq .05$). Visceral adipose tissue area was the best correlate of CD68+ cell percentage in both omental and subcutaneous fat tissues, explaining, respectively, 20% and 12% of the variance in models also including subcutaneous adipose tissue area, adipocyte sizes, and total body fat mass. Visceral adipose tissue accumulation is the best correlate of macrophage infiltration in both the subcutaneous and omental fat compartments of lean to obese women.

1. Introduction

Obesity is associated with chronic, low-grade inflammation [1], a condition that has been proposed as a potential mechanism linking obesity, insulin resistance, and type 2 diabetes mellitus [1,2]. Increased body fat mass has been associated with elevations in systemic inflammatory mediators such as C-reactive protein (CRP), tumor necrosis factor

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(TNF)–α, monocyte chemoattractant protein (MCP)–1, and interleukin (IL)-6 [3-5].

Human and animal studies have now shown that obesity is associated with increased macrophage infiltration in adipose tissue [6-8]. Cellular antigen CD68 has been used as a macrophage marker to show that these cells can be detected on slides from human adipose tissues [9]. Adipose tissue macrophages (ATMs) are essentially derived from bone marrow [6] and may be recruited in response to necrotic adipocytes and/or proinflammatory cytokines produced locally [10,11]. Adipose tissue macrophages of massively obese women typically aggregate in ring patterns or crownlike structures around adipocytes in the subcutaneous adipose tissue depot, suggesting that they may have phagocytic activity [10].

Accumulation of adipose tissue in the abdominal cavity has been closely associated with metabolic alterations related to obesity [12,13], and regional differences in macrophage infiltration in adipose tissue may have a crucial role in the pathophysiology of the comorbidities related to visceral obesity [14,15]. However, only a few studies have examined regional differences in ATM infiltration. Recently, visceral obesity was associated with increased macrophage infiltration in omental fat of obese subjects. Cancello et al [8] have shown that macrophage infiltration was higher in omental than in subcutaneous adipose tissue and was significantly associated with severe hepatic fibroinflammatory lesions in severely obese individuals. Another study observed increased macrophage infiltration in omental compared with subcutaneous fat in lean to obese subjects. This was exacerbated in subjects with higher intraabdominal adipose tissue accumulation. In the same study, preferential macrophage infiltration in omental adipose tissue was observed in a subgroup of women with impaired glucose homeostasis [16]. The number of proinflammatory macrophages (M1) was also increased in omental compared with subcutaneous fat tissues in obese women [17]. In contrast, a recent study has shown that proinflammatory macrophages (CD11c+ cells) were more abundant in subcutaneous than in omental adipose tissue, a finding that correlated with markers of insulin resistance in obese women [18]. Thus, the extent and impact of regional differences in ATM infiltration remain unclear in the lean to moderately obese range of adiposity.

Hence, the aim of this study was to examine regional differences in ATM infiltration and its relationship with obesity-related complications in lean to obese women. Given its well-documented link with adipose tissue dysfunction, we tested the hypothesis that visceral obesity is a significant correlate of macrophage infiltration in abdominal adipose tissues of women.

2. Subjects and methods

2.1. Subjects

The study sample included 40 healthy women aged 37.6 to 54.3 years who were recruited through the elective surgery schedule of the Gynecology Unit at Laval University Medical Center. Women of the study elected for subtotal (n = 1) or total (n = 39) abdominal hysterectomies, some with salpingo-oophorectomy of 1 (n = 4) or 2 (n = 11) ovaries. Reasons for surgery included one or more of the following: menorrhagia/menometrorrhagia (n = 17), myoma/fibroids (n = 36), incapacitating dysmenorrhea (n = 3), pelvic pain (n = 2), benign cyst (n = 8), endometriosis (n = 5), pelvic adhesions (n = 1), hypermenorrhea (n = 1), excessive anaemia-causing uterine bleeding (n = 1), endometrial hyperplasia (n = 3), or polyp (n = 3). This study was approved by the Research Ethics committee of Laval University Medical Center. All subjects provided written informed consent before their inclusion in the study.

2.2. Body fatness and body fat distribution measurements

These tests were performed a few days before the surgery. Measures of total body fat mass, fat percentage, and fat-free mass were determined by dual-energy x-ray absorptiometry using a Hologic QDR-2000 densitometer and the enhanced array whole-body software V5.73A (Hologic, Bedford, MA). Measurement of abdominal subcutaneous and visceral adipose tissue cross-sectional areas was performed by computed tomography as previously described [19] using a GE Light Speed 1.1 CT scanner (General Electric Medical Systems, Milwaukee, WI). Subjects were examined in the supine position, with arms stretched above the head. The scan was performed at the L4-L5 vertebrae level using a scout image of the body to establish the precise scanning position. The quantification of visceral adipose tissue area was done by delineating the intraabdominal cavity at the internalmost aspect of the abdominal and oblique muscle walls surrounding the cavity and the posterior aspect of the vertebral body using the ImageJ 1.33u software (National Institutes of Health, Bethesda, MD). Subcutaneous adipose tissue area was obtained by subtracting visceral area from total adipose tissue area at L4-L5. Adipose tissue areas were highlighted and computed using an attenuation range of –190 to –30 Hounsfield units. The coefficient of variation between 2 measures from the same observer (n = 10) were 0.0%, 0.2%, and 0.5% for total, subcutaneous, and visceral adipose tissue areas, respectively.

2.3. Plasma lipid-lipoprotein measurements

Blood samples were obtained after a 12-hour fast on the morning of surgery. Cholesterol and triglyceride level measurements in plasma and lipoprotein fractions were performed with a Technicon RA analyzer (Bayer, Etobicoke, Ontario, Canada) using enzymatic methods, as previously described [20]. Plasma very low-density lipoproteins (VLDLs) were isolated by ultracentrifugation, and the high-density lipoprotein (HDL) fraction was obtained by precipitation of low-density lipoproteins (LDLs) from the infranatant with heparin and $MnCl_2$ [21]. The cholesterol content of the infranatant was measured before and after precipitation, and the concentration of LDL cholesterol was obtained by difference. Apolipoprotein (Apo) B and Apo A-1 concentrations were measured using the rocket immunoelectrophoretic method of Laurell [22] as described previously [20].

2.4. Glucose homeostasis and inflammatory markers

Fasting glucose and insulin were measured in presurgery blood samples. Plasma glucose was measured using the glucose oxidase method, and plasma insulin levels were measured by radioimmunoassay (Linco Research, St Charles, MO). The homeostasis model assessment (HOMA) insulin resistance index was calculated [23]. Plasma IL-6 levels were measured by a commercially available enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). Plasma CRP levels were measured in plasma using the Behring Latex-Enhanced highly sensitive CRP assay on a Behring Nephelometer BN-100 (Behring Diagnostic, Westwood, MA) and the calibrators (N Rheumatology Standards SL) provided by the manufacturer.

2.5. Adipose tissue sampling

Subcutaneous and omental adipose tissue samples were, respectively, collected at the site of the surgical incision (lower abdomen) and at the distal portion of the greater omentum and immediately carried to the laboratory. A portion of the fresh sample was used for adipocyte isolation. Immediately following surgical removal, fresh adipose tissue samples were fixed in 10% formalin for 24 to 48 hours at room temperature prior to processing for routine paraffin wax embedding.

2.6. Adipocyte isolation

Fresh tissue samples were digested with collagenase type I in Krebs-Ringer-Henseleit buffer for 45 minutes at 37°C according to a modified version of the Rodbell method [24]. Adipocyte suspensions were filtered through nylon mesh and washed 3 times with Krebs-Ringer-Henseleit buffer. For cell size measurements, mature adipocyte suspensions were visualized using a contrast microscope attached to a camera and computer interface. Pictures of cell suspensions were taken, and the Scion Image software was used to measure the size (diameter) of 250 adipocytes per tissue sample. Average adipocyte size was used in analyses.

2.7. Immunohistochemistry

Five-micrometer slices of subcutaneous and omental adipose tissues were mounted on the same slide. Mounted slides were deparaffinized in toluene and rehydrated through graded ethanol 95%, 75%, and 50%. Tissue sections were incubated with 3% hydrogen peroxide in methanol for 30 minutes to block endogenous activity and subsequently rinsed with ethanol and distilled water. Antigen retrieval was carried out in 0.1 mol/L sodium citrate buffer by boiling slides in a microwave oven for 2 minutes at maximum power and then cooling them down at room temperature. Slides were rinsed once in distilled water for 5 minutes and once in phosphate-buffered saline (PBS) for 5 minutes before the blocking step.

Unspecific binding was blocked by incubation of tissue sections in 10% goat serum in PBS containing 0.4% Triton X-100 and 0.1% bovine serum albumin for 30 minutes. The tissue sections were incubated with the primary antibody overnight at 4°C. The mouse anti-human CD68 antibody (Cedarlane, Burlington, Ontario, Canada) was used in a dilution of 1:50. Slides were then washed 4 times with PBS. The goat anti-mouse antibody coupled with Alexa Fluor 594 (Dako Canada, Mississauga, Ontario, Canada) diluted 1:200 was used in the coupling solution (blocking solution) for 2 hours at room temperature. Slides were washed 4 times with PBS and mounted in Vectashield Mounting Medium containing 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to visualize whole cells. Blue (DAPI) and red fluorescence (immunoreactivity) was visualized using a fluorescence microscope. The number of cells infiltrated by macrophages was counted in a blinded manner. A minimum of 400 adipocytes was examined for each sample. For each subject, the number of macrophages (identified as CD68+ cells) and crownlike structures within the entire tissue section was counted. A *crownlike structure* was defined as an adipocyte completely surrounded by macrophages. The number of macrophages and the number of crownlike structures were normalized for 100 adipocytes (percentage macrophages) for comparison among tissue samples and women.

2.8. Messenger RNA expression by quantitative real-time reverse transcriptase polymerase chain reaction

Total RNA was isolated from whole subcutaneous and omental adipose tissue using the RNeasy lipid tissue extraction kit and on-column DNase treatment (Qiagen, Valencia, CA) following the manufacturer's recommendations. RNA quality and concentration were assessed using the Agilent Technologies 2100 Bioanalyzer (Agilent, Palo Alto, CA). Complementary DNA was generated from 4 μg of total RNA with 50 ng of random hexamers, 300 ng of oligo dT_{18}, and 200 U of Superscript II Rnase H-RT (Invitrogen Life Technologies, Carlsbad, CA) and purified with QIAquick PCR Purification Kit (Qiagen). Real-time complementary DNA amplification was performed in duplicate using the LightCycler 480 (Roche Diagnostics, Indianapolis, IN) and the SYBR Green I Master (Applied Biosystems, Foster City, CA) as follows: 95°C for 10 seconds, 60°C to 62°C for 10 seconds, 72°C for 14 seconds, and then 76°C for 5 seconds (reading) repeated 45 times. Target gene amplifications were normalized using expression levels of ATP synthase O subunit (*ATP5O*) as the housekeeping gene. Expression levels of this gene were not associated with age and adiposity in our study sample. Similar results were obtained using other housekeeping genes. Only results normalized to *ATP5O* expression are shown in the present article. Primer sequences for CD68 (NM_001251; sense: 5′-GCAGCAACTCGAGCATCATTCTT-3′; anti-sense: 5′-CGAGGAGGCCAAGAAGGATCA-3′), CD11b (NM_000632; sense: 5′-TTCCAGAACAACCCTAACCCAAGATC-3′; anti-sense: 5′-ATCGCCAAACTTTTCTCCATCCG-3′), CD11c (NM_000887; sense: 5′-GGCCATGCACAGATACCAGGT-3′; anti-sense: 5′-CTGGGGGTGCGATTTTCTCTG-3′), and CD40 (NM_001250; sense: 5′-GGCACCTCAGAAACAGACACCAT-3′; anti-sense: 5′-ACATTGGAGAAGAAGCCGACTG-3′) were designed using GeneTools (Biotools, Jupiter, FL).

2.9. Statistical analyses

Student paired *t* tests were performed to identify depot differences in adipose tissue CD68, CD11b, CD11c, and CD40

messenger RNA (mRNA) expression levels as well as CD68+ cell percentage. Pearson correlation coefficients were computed to quantify associations between CD68+ cell percentage, expression of macrophage-specific gene markers, and adiposity measurements. Statistical adjustments for total body fat mass and visceral adipose tissue area were performed using covariance analysis. Analysis of variance was used to compare mean CD68+ cell percentage and mean CD68 expression levels in omental and subcutaneous adipose tissue of lean, overweight, and obese women. Multivariate linear regressions were computed to identify the best correlate of CD68+ cells and macrophage-specific gene markers in both the omental and subcutaneous adipose tissues with models including visceral adipose tissue area, subcutaneous adipose tissue area, omental adipocyte diameter, subcutaneous adipocyte diameter, and total body fat mass. Log_{10} and Box-Cox transformations were used for nonnormally distributed variables. Statistical analyses were performed with the JMP 4.0 software (SAS Institute, Cary, NC).

3. Results

Subcutaneous and omental adipose tissue CD68+ cell percentage and expression of macrophage specific markers were measured in a sample of 40 women. Characteristics of the study women are shown in Table 1. Women were approximately 47 years old and were overweight with an average body mass index (BMI) of 28.4 kg/m^2. The range of adiposity covered the spectrum from lean to obese, with BMI values spanning from 19.9 to 50.1 kg/m^2.

Table 1 – Characteristics of the 40 women of the study

Variables	Mean ± SD	Range (min-max)
Age (y)	47.0 ± 3.9	37.6-54.3
Weight (kg)	74.5 ± 16.0	53-133
Waist circumference (cm)	95.6 ± 13.6	73-147
BMI (kg/m^2)	28.4 ± 5.8	19.9-50.1
Total body fat mass (kg)	27.7 ± 7.8	12.4-43.6
Adipose tissue area (cm^2)		
Total	439 ± 135	192-773
Subcutaneous	336 ± 114	129-555
Visceral	103 ± 55	45-266
Adipocyte diameter (μm)		
Subcutaneous	103.8 ± 12.2	75-131
Omental	89.3 ± 13.9	67-121
Plasma lipid profile		
Cholesterol (mmol/L)		
Total	5.36 ± 0.97	3.53-6.92
VLDL	0.50 ± 0.28	0.13-1.22
LDL	3.42 ± 0.81	1.87-4.69
HDL	1.43 ± 0.32	0.75-2.16
Triglycerides (mmol/L)		
Total	1.47 ± 0.63	0.67-2.96
VLDL	0.97 ± 0.54	0.29-2.16
LDL	0.26 ± 0.10	0.11-0.57
HDL	0.25 ± 0.04	0.19-0.34
Apo (g/L)		
Total Apo B	1.03 ± 0.23	0.55-1.55
HDL–Apo A-1	1.43 ± 0.22	0.99-1.86
Cholesterol–HDL cholesterol	3.89 ± 0.99	2.37-6.25
Glucose homeostasis		
Fasting glucose (mmol/L)	5.9 ± 0.7	4.4-7.1
Fasting insulin (μU/mL)	11.7 ± 7.6	3.3-45.3
HOMA index	3.2 ± 2.3	0.7-13.7
Plasma inflammatory markers		
IL-6 (pg/ml) [a]	1.18 ± 0.88	0.28-3.49
CRP (mg/L) [a]	2.25 ± 2.15	0.17-8.97

[a] n = 38.

3.1. *Regional differences in ATM infiltration*

Fig. 1 shows representative fluorescence staining of CD68+ macrophages in omental and subcutaneous fat from a lean woman (Fig. 1A-B) vs an obese woman (Fig. 1C-D). The total number of infiltrating macrophages was systematically counted on several slides and presented as the number of CD68+ cells per 100 adipocytes (percentage of macrophages). CD68 mRNA expression and CD68+ cell percentage in whole adipose tissue samples are shown in Fig. 2. Mean CD68+ cell percentage tended to be higher in subcutaneous compared with omental adipose tissue ($P = .07$) (Fig. 2A). There was no significant depot difference in CD68 mRNA abundance (Fig. 2B). CD68+ cell percentage in the omental fat compartment was positively and significantly correlated with CD68+ cell percentage in subcutaneous adipose tissue ($P = .0001$) (Fig. 2C). A significant positive correlation was also observed between CD68 mRNA expression in omental tissue and CD68 mRNA expression in subcutaneous tissue ($P \leq .01$) (Fig. 2D). We subdivided the women in 3 subgroups according to BMI categories of the World Health Organization. In obese women (n = 13), we observed that mean CD68+ cell percentage tended to be higher in subcutaneous adipose tissue compared with omental adipose tissue ($P < .10$) (Fig. 2E). No significant depot difference was observed in CD68+ cell percentage in lean (n = 12) and overweight women (n = 15). In a similar manner, no significant depot difference in CD68 mRNA expression of lean, overweight, and obese women was observed (Fig. 2F). Mean CD68+ cell percentage in both adipose compartments tended to be higher in obese women compared with lean women ($P < .10$). Significantly higher CD68 mRNA expression levels were observed in both fat compartments of obese women compared with lean women ($P \leq .05$). Furthermore, we observed significantly higher CD68 mRNA expression in subcutaneous adipose tissue of overweight women compared with lean women ($P \leq .05$).

A trend for a correlation between CD68+ cell percentage in subcutaneous adipose tissue and CD68 mRNA expression in this depot was found ($P = .08$). No significant association was observed between omental CD68+ cell percentage and omental CD68 mRNA expression (data not shown). In the whole sample, only 8 women had ATMs typically dispersed as crownlike structures. We identified 6 women with at least one crownlike structure in subcutaneous adipose tissue and 4 women with at least one crownlike structure in omental adipose tissue. The percentage of crownlike structures in these women was very low, varying between 0.08% and 0.49% (data not shown). All the women with crownlike structures in subcutaneous adipose were obese (BMI between 31 and 50 kg/m^2).

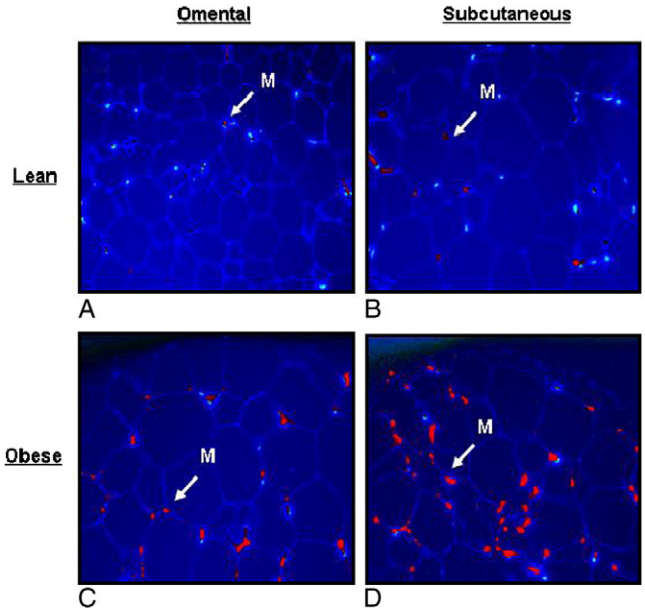

Fig. 1 – Representative fluorescence immunohistochemical detection of CD68+ macrophages in omental and subcutaneous fat samples from a lean woman and an obese woman, selected according to their BMI value. (A) Omental and (B) subcutaneous fat from a lean woman; (C) omental and (D) subcutaneous fat from an obese woman. M indicates macrophage. Slides were counterstained with DAPI (in blue), and red fluorescence (CD68+ cells) was visualized using a fluorescence microscope. Adipocytes and CD68+ cells were counted in a blinded manner. A minimum of 400 adipocytes was examined for each sample.

There was no significant depot difference in CD11b mRNA abundance. However, mean CD11c mRNA expression was significantly higher in subcutaneous compared with omental adipose tissue ($P \leq .01$). In contrast, mean CD40 mRNA abundance was significantly higher in omental compared with subcutaneous adipose tissue ($P \leq .01$). CD11c mRNA abundance in the omental fat compartment was positively correlated with CD11c mRNA abundance in subcutaneous adipose tissue ($P = .00001$). A significant positive association was also observed between CD40 mRNA expression in omental adipose tissue and CD40 mRNA expression in subcutaneous adipose tissue ($P \leq .01$) (data not shown).

3.2. *ATM infiltration in relation with body fatness and body fat distribution*

Table 2 shows correlation coefficients between CD68+ cell percentage or CD68 mRNA expression in both adipose tissue depots and adiposity measurements. Weight, BMI, and waist circumference were positively associated with CD68+ cell percentage and CD68 mRNA expression in both adipose tissue compartments ($P \leq .05$). Visceral adipose tissue area and omental adipocyte diameter were positively related to CD68+ cell percentage in omental fat ($P \leq .05$). Total and visceral adipose tissue areas as well as subcutaneous adipocyte diameter were significantly correlated with CD68 + cell percentage in subcutaneous adipose tissue ($P \leq .05$). CD68 mRNA expression in omental adipose tissue tended to be correlated with total and subcutaneous adipose tissue areas ($P < .10$). Adipose tissue areas and subcutaneous adipocyte diameter were significantly associated with CD68 mRNA expression in the subcutaneous fat compartment ($P \leq .05$).

Table 3 shows Pearson correlation coefficients between CD11b or CD11c mRNA expression in both adipose tissue depots and adiposity measurements. Weight, BMI, and waist circumference were positively associated with CD11b mRNA expression in subcutaneous adipose tissue and CD11c mRNA expression in both adipose tissue compartments ($P \leq .05$). Adipose tissue areas and adipocyte diameters were positively related to CD11b and CD11c in subcutaneous adipose tissue ($P \leq .05$). No significant association was observed between CD40 mRNA expression in either adipose tissue compartment and measurements of body fatness and body fat distribution (data not shown).

To identify the strongest correlates of ATM infiltration in both depots, we generated multiple linear regression models that included visceral adipose tissue area, subcutaneous adipose tissue area, omental adipocyte diameter, subcutaneous adipocyte diameter, and total body fat mass. Visceral adipose tissue area was the best correlate of CD68+ cell percentage in both the omental and subcutaneous depots, explaining, respectively, 20% ($P = .008$) and 12% ($P = .04$) of the variance. Regarding mRNA measurements, omental adipocyte diameter was the best correlate of CD11b and CD11c mRNA expression in subcutaneous adipose tissue, explaining, respectively, 12% ($P = .009$) and 23% of the variance. Omental adipocyte diameter was also a significant correlate of CD68 expression in the subcutaneous fat depot and CD11b expression in omental adipose tissue, explaining both 5% ($P = .05$) of the variance (data not shown).

3.3. *ATM infiltration in relation with plasma inflammation markers*

CD68 mRNA expression in both fat depots was positively correlated with plasma IL-6 concentrations ($P \leq .05$) and tended to be positively associated with CRP concentrations (Table 2). No significant association was found between CD68+ cell percentage in either depot and plasma IL-6 levels or high-sensitivity CRP concentrations. CD11b mRNA expression in subcutaneous fat was also positively correlated with plasma IL-6 concentrations and high-sensitivity CRP concentrations ($P \leq .05$) (Table 3). CD11c mRNA abundance in subcutaneous adipose tissues tended to be positively associated with IL-6 concentrations ($P = .07$).

3.4. *ATM infiltration in relation with metabolic alterations*

Table 4 shows Pearson correlation coefficients between CD68+ cell percentage in each adipose tissue depot and blood lipid profile or glucose homeostasis measurements. CD68+ cell percentage in the omental fat compartment was negatively associated with HDL cholesterol as well as HDL–Apo A-1 ($P \leq .05$). Higher CD68+ cell percentage in omental adipose tissue

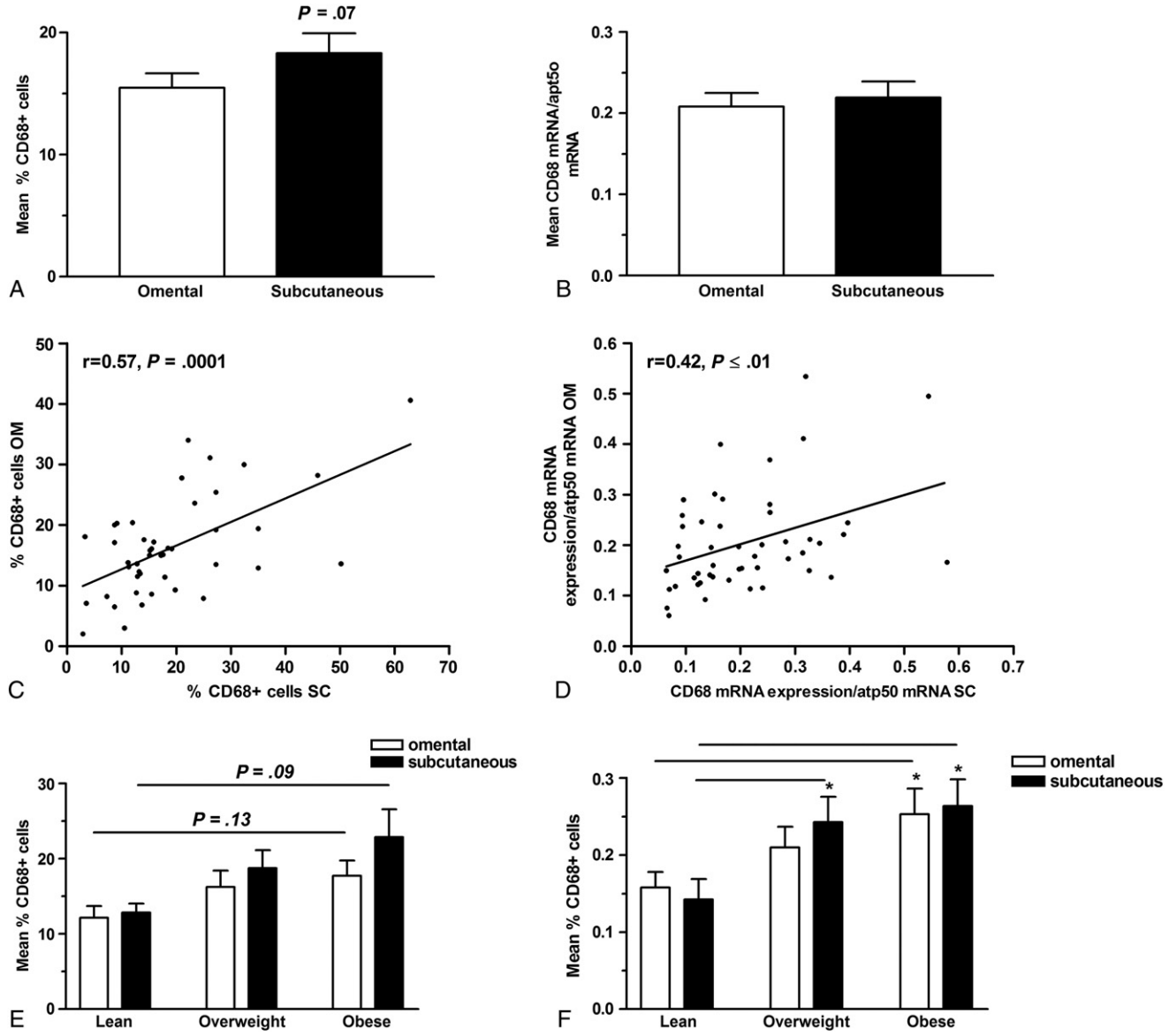

Fig. 2 – (A) Percentage of CD68+ cells in omental vs subcutaneous adipose tissue. (B) Omental vs subcutaneous CD68 mRNA expression. (C) Correlation between CD68+ cell percentage in the omental compartment and CD68+ cell percentage in subcutaneous adipose tissue. (D) Correlation of CD68 mRNA abundance in the omental vs subcutaneous compartment. (E) Mean CD68+ cell percentage in omental and subcutaneous adipose tissue of lean, overweight, and obese women. (F) Mean CD68 mRNA expression in omental and subcutaneous adipose tissue of lean, overweight, and obese women; Pearson correlation coefficients are shown. Correlation coefficients and *P* values were computed with normalized variables (log_{10} and Box-Cox transformations).

was associated with higher values of VLDL-triglycerides, the cholesterol to HDL cholesterol ratio, fasting insulin, and HOMA index ($P \leq .05$). Positive trends were observed between CD68+ cell percentage in the omental fat compartment and concentrations of triglycerides, VLDL-cholesterol, and fasting glucose ($P \leq .10$). Higher CD68+ cell percentage in the subcutaneous fat compartment was associated with lower concentrations of HDL–Apo A-1 and higher fasting glucose concentrations ($P \leq .05$). After adjustment for total body fat mass, only HDL cholesterol remained significantly associated with CD68+ cell percentage in the omental fat compartment ($P \leq .05$). However, CD68+ cell percentage in either fat depot was no longer associated with measures of the blood lipid profile and glucose homeostasis following adjustment for visceral adipose tissue area.

4. Discussion

Several lines of evidence have demonstrated that macrophage infiltration in adipose tissue is a feature of the inflammatory state associated with obesity. The objective of our study was to examine regional differences in ATM infiltration and its relationship with obesity and related complications in lean to obese women. We tested the hypothesis that visceral obesity would be a correlate of macrophage infiltration at least in

Table 2 – Pearson correlation coefficients between CD68+ cells percentage, CD68 mRNA expression level [a] in subcutaneous or omental adipose tissue, and characteristics of the sample (n = 40)

Variables	% CD68+ cells				CD68 mRNA expression [a]			
	SC		OM		SC		OM	
	r	P	r	P	r	P	r	P
Anthropometrics								
Weight	0.34	.03	0.39	.01	0.62	.0001	0.48	.001
BMI	0.31	.05	0.44	.004	0.57	.0001	0.39	.01
Waist circumference [b]	0.37	.02	0.47	.002	0.56	.0002	0.41	.01
Total body fat mass [c]	0.24	.14	0.28	.09	0.55	.0003	0.30	.07
Adipose tissue areas								
Total [c]	0.32	.05	0.35	.03	0.49	.001	0.26	.12
Visceral [b]	0.40	.01	0.52	.0006	0.49	.001	0.20	.21
Subcutaneous [c]	0.24	.15	0.22	.18	0.43	.006	0.26	.12
Adipocyte diameter								
Omental [c]	ND		0.35	.03	ND		0.08	.62
Subcutaneous [b]	0.39	.02	ND		0.48	.002	ND	
Plasma inflammatory markers								
IL-6 [c]	0.03	.86	0.12	.46	0.40	.02	0.33	.05
C-reactive protein [c]	0.11	.49	0.19	.25	0.28	.10	0.28	.09

SC indicates subcutaneous; OM, omental; ND: not determined.
[a] Expression levels relative to *ATP5O* mRNA expression.
[b] n = 39.
[c] n = 38.

omental adipose tissue. CD68+ macrophages tended to be more abundant in subcutaneous compared with omental adipose tissue, and no significant regional difference in CD68 mRNA expression was found. We also observed strong positive correlations of CD68+ cell percentage as well as CD68 mRNA expression in omental vs subcutaneous adipose tissue. As expected from previous literature, most measures of total adiposity and body fat distribution were positively correlated with CD68+ cell percentage or expression of macrophage-specific gene markers (CD68, CD11b, and CD11c) in subcutaneous and/or omental adipose tissue. As shown with our regression analyses, we report for the first time that visceral adipose tissue area is an independent and significant correlate of CD68+ cell percentage in both omental and subcutaneous fat. Moreover, CD68+ cell percentage in both fat depots was no longer associated with blood lipid alterations and glucose homeostasis following adjustment for visceral adipose tissue area.

The notion that macrophage infiltration is predominant in visceral fat has been put forward in the literature [15]. This is supported by some reports. One study has shown that macrophage infiltration (HAM56+ cell percentage) was greater in omental than in subcutaneous adipose tissue in severely obese subjects [8]. Moreover, in both men and women, another study has observed increased macrophage infiltration (CD68+ cell percentage) in omental compared with subcutaneous fat in lean to obese subjects; and this was exacerbated in obese subjects with visceral obesity [16]. The number of proinflammatory macrophages (M1) was also increased in omental compared with subcutaneous adipose tissue in obese women [17]. We rather demonstrate that mean CD68+ cell percentage tended to be higher in subcutaneous compared with omental adipose tissue. We suggest that this finding is possibly due to the characteristics of the sample we examined. Our study included a group of lean to obese women, whereas samples of other studies were composed of obese or severely obese men and women. Our population was apparently characterized by a lower inflammatory state compared with severely obese subjects. This hypothesis is supported by the fact that only 12 women had CRP values higher than 3.0 mg/dL and that the vast majority had a normal metabolic profile. Our results would possibly have been comparable to those of others if participants tested had been more obese. Regarding other markers examined, no significant depot difference in mRNA abundance of CD68 and CD11b was observed. Furthermore, mean CD11c mRNA expression was significantly higher in the subcutaneous vs the omental fat depot. In contrast, CD40 mRNA abundance was significantly higher in omental compared with subcutaneous adipose tissue. Similar to our findings, a recent study has shown that proinflammatory macrophages (CD11c+ cells) were more abundant in subcutaneous compared with omental adipose tissue of both obese and postobese women [18]. Moreover, also consistent with our findings, another study observed that the number of CD40+ macrophages was significantly higher in omental compared with subcutaneous adipose tissue in obese women [17]. In light of these results, depot differences in macrophage infiltration seem to differ as a function of the macrophage gene markers examined.

Our results show for the first time that visceral adipose tissue area is a strong correlate of CD68+ cell percentage in

Table 3 – Pearson correlation coefficients between CD11b [a] or CD11c [a] mRNA expression in SC or OM adipose tissue and characteristics of the sample (n=40)

Variables	CD11b mRNA expression [a]				CD11c mRNA expression [a]			
	SC		OM		SC		OM	
	r	P	r	P	r	P	r	P
Anthropometrics								
Weight	0.54	.0003	0.16	.26	0.36	.02	0.30	.05
BMI	0.55	.0002	0.12	.40	0.37	.01	0.29	.05
Waist circumference [b]	0.59	.0001	0.14	.35	0.43	.005	0.31	.04
Total body fat mass [c]	0.56	.0002	0.24	.10	0.36	.02	0.22	.17
Adipose tissue areas								
Total [c]	0.57	.0001	0.21	.17	0.35	.03	0.25	.12
Visceral [b]	0.38	.01	−0.07	.63	0.36	.02	0.24	.14
Subcutaneous [c]	0.54	.0004	0.28	.07	0.32	.05	0.19	.24
Adipocyte diameter								
Omental [c]	0.54	.0003	−0.27	.16	0.49	.001	0.23	.14
Subcutaneous [b]	0.42	.008	0.06	.67	0.37	.02	0.18	.26
Plasma inflammatory markers								
IL-6 [c]	0.41	.005	−0.04	.79	0.29	.07	0.18	.26
CRP [c]	0.42	.003	−0.18	.21	0.11	.47	−0.01	.93

[a] Expression levels relative to *ATP5O* mRNA expression.
[b] n = 39.
[c] n = 38.

Table 4 – Pearson correlations between CD68+ cell percentage in SC or OM adipose tissue and measures of the blood lipid profile or glucose homeostasis (n = 40)

Variables	% CD68+ cells					
	Unadjusted		Adjusted for TBFM		Adjusted for VAT	
	SC	OM	SC	OM	SC	OM
Lipid profile						
Cholesterol [b]						
Total	–0.04	–0.10	–0.10	–0.09	–0.10	–0.17
VLDL	0.23	0.28 †	0.20	0.23	0.01	0.07
LDL	–0.07	–0.06	–0.14	–0.07	–0.17	–0.17
HDL	–0.26	–0.42 *	–0.20	–0.36 *	–0.02	–0.21
Triglycerides [b]						
Total	0.18	0.29 †	0.13	0.24	–0.04	0.08
VLDL	0.20	0.33 *	0.13	0.27	–0.03	0.12
LDL	0.08	0.18	0.04	0.10	–0.06	0.05
HDL	–0.02	–0.10	–0.006	–0.08	–0.06	–0.18
Apo [b]						
Total Apo B	0.02	0.13	–0.07	0.09	–0.15	–0.03
HDL–Apo A-1	–0.36 *	–0.39 *	–0.29 †	–0.32 †	–0.23	–0.27
Total cholesterol–HDL cholesterol [b]	0.21	0.34 *	0.12	0.18	–0.06	0.08
Glucose homeostasis						
Fasting glucose [a]	0.31 *	0.28 †	0.20	0.18	0.23	0.23
Fasting insulin [a]	0.21	0.31 *	0.03	0.10	0.04	0.17
HOMA index [a]	0.24	0.33 *	0.06	0.13	0.08	0.19

TBFM indicates total body fat mass; VAT, visceral adipose tissue area.
[a] n = 39.
[b] n = 38.
* $P \leq .05$.
† $P \leq .08$.

both adipose tissues independent of adipocyte sizes, subcutaneous adipose tissue area, and total body fat mass. Omental adipocyte diameter was also the best correlate of CD68, CD11b, and CD11c mRNA expression in subcutaneous adipose tissue independent of subcutaneous adipocyte size, adipose tissue areas, and total body fat mass. The recruitment and activation of macrophages in adipose tissue are probably multifactorial [25]. Some studies have suggested that expanding adipose tissue might produce chemotactic signals such as monocyte chemotactic protein (MCP)-1 and colony stimulating factor (CSF)-3, which lead to macrophage recruitment [8,11]. Adipocyte hypertrophy creating local hypoxic conditions may also be involved in the attraction of macrophages by stimulating inflammatory pathways such as JNK1-regulated chemokine release [6,15,26,27]. Cinti et al [10] also hypothesized that death of hypertrophied fat cells might be a stimulus regulating ATM accumulation. Furthermore, Kosteli et al [28] suggested that excess lipids may be a potential and central regulator of ATM accumulation. Taken together, our results suggest that excess visceral fat accumulation, possibly with hypertrophic adipocytes, is a good marker of these local alterations. This is consistent with the notion that visceral obesity is a marker of dysfunctional adipose tissue [29].

Previous studies have already shown that subcutaneous and/or visceral ATMs increase with the degree of adiposity [6-8,10,16,30], which is highly consistent with our results. For example, Weisberg et al [6] found that the percentage of cells expressing the macrophage marker F4/80 in subcutaneous fat was significantly and positively correlated with adipocyte size and body fat mass in humans. Harman-Boehm et al [16] demonstrated that ATM counts correlated with clinical parameters of obesity such as BMI, waist circumference, fasting insulin levels, and systolic blood pressure in both adipose tissue depots. These results are consistent with those presented in our study. We show that the associations between macrophage infiltration and metabolic parameters are explained by higher visceral adipose tissue accumulation and omental adipocyte hypertrophy. As previously discussed, the role of ATM accumulation in the pathophysiology of human obesity and related complications is still unclear [15]. Our study demonstrates that CD68+ cell percentages in both fat depots are no longer related to measures of the lipid profile and glucose homeostasis following adjustment for visceral adipose tissue area. The association between insulin resistance and ATMs is very clear in obese mice [7,31]. However, consistent with our results, previous human studies did not find any correlation between total ATM infiltration (CD68+ cells) and insulin resistance after adjusting for body fat mass, age, or sex [8,16,32].

Several studies have reported significant correlations between visceral adipose tissue accumulation and circulating levels of IL-6 and CRP [33-35]. Macrophages in human adipose tissue may contribute to this obesity-related chronic, low-grade inflammatory state [1,15] through increases in systemic concentrations of proinflammatory cytokines. We found that higher plasma IL-6 and CRP levels were associated with higher CD68 mRNA expression in both adipose tissues, as seen previously [32]. CD11b mRNA expression in the subcutaneous fat depot was also positively correlated with plasma IL-6 concentrations and high-sensitivity CRP concentrations. The lack of significant association between CD68+ cell percentage and plasma IL-6 concentrations or plasma CRP levels is possibly due to the mild inflammatory state in our sample. We only observed a very small number of women with adipose tissue-infiltrating macrophages typically dispersed as crownlike structures. Thus, ringlike accumulation patterns may represent more advanced stages of the inflammatory response that would be more likely to relate to systemic inflammation assessed by higher plasma CRP levels [36]. Studies have also demonstrated that IL-6 secreted by adipose tissue may account for a third of plasma cytokine levels [5,37,38]. Thus, plasma concentration of inflammatory markers may only partially reflect local ATM infiltration, as previously suggested by Cancello and Clement [25].

In the present study, CD68 labeling was used to identify total macrophage infiltration. CD68+ cells have been described as resident cells at the junction of 2 or more adipocytes [18]. Recently, obesity has been associated with changes in macrophage activation state, from the alternative (M2) to the classic (M1) macrophages phenotype. M1 macrophages are described as proinflammatory and characterized by higher secretion of TNF-α, IL-6, and MCP-1, whereas M2 macrophages are described as anti-inflammatory and characterized by higher expression of IL-10 [39]. One study has shown that M1 phenotype macrophages were more abundant in subcutaneous adipose tissue from obese

women compared with lean controls and that the M1/M2 ratio decreased after weight loss induced by gastric bypass [17]. Our study indirectly examined various macrophage activation states through the use of CD68, CD11b, CD11c, and CD40 expression. These markers have generated slightly divergent results regarding depot differences, but had relatively similar correlations with adiposity and metabolic alterations, with the exception of CD40. Furthermore, a trend for a correlation between CD68+ cell percentage in subcutaneous adipose tissue and CD68 mRNA expression in this depot was found. Nonnormalized subcutaneous CD68 mRNA levels were even significantly related to CD68+ cell percentage in this fat depot. However, no significant association was observed between CD68+ cell percentage and CD68 mRNA expression in omental fat. We suggest that CD68 mRNA expression seems to represent a moderately reliable measure of CD68 cell infiltration, at least in subcutaneous adipose tissue. Additional studies are required to explain the lack of correlation between CD68 mRNA and CD68+ cell percentage in the omental fat compartment. Other limitations of the study should be acknowledged. These results are cross-sectional, which prevents us for concluding on cause-and-effect relationships. Our study also did not include men. Indeed, we examined only women because of the difficulty of setting up similar studies including lean to moderately obese men.

In summary, as opposed to previous literature in severely obese patients, macrophage infiltration was not more abundant in omental fat compared with the subcutaneous fat depot in the lean to obese range in women. Percentages of macrophage infiltration in both fat depots were not associated with markers of systemic inflammation and were no longer related to measures of the lipid profile and glucose homeostasis after adjustment for visceral adipose tissue area. However, this study demonstrated that visceral adipose tissue accumulation is an indicator of macrophage infiltration in both the subcutaneous and omental fat compartments of lean to obese women.

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Conflict of Interest

No author declared a conflict of interest.

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