Macrophage Content in Subcutaneous Adipose Tissue

Associations With Adiposity, Age, Inflammatory Markers, and Whole-Body Insulin Action in Healthy Pima Indians

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OBJECTIVE—In severely obese individuals and patients with diabetes, accumulation and activation of macrophages in adipose tissue has been implicated in the development of obesity-associated complications, including insulin resistance. We sought to determine whether in a healthy population, adiposity, sex, age, or insulin action is associated with adipose tissue macrophage content (ATMc) and/or markers of macrophage activation.

RESEARCH DESIGN AND METHODS—Subcutaneous ATMc from young adult Pima Indians with a wide range of adiposity (13–46% body fat, by whole-body dual-energy X-ray absorptiometry) and insulin action (glucose disposal rate 1.6–9 mg/kg estimated metabolic body size/min, by glucose clamp) were measured. We also measured expression in adipose tissue of factors implicated in macrophage recruitment and activation to determine any association with ATMc and insulin action.

RESULTS—ATMc, as assessed by immunohistochemistry (Mphi) and by macrophage-specific gene expression (CD68, CD11b, and CSF1R), were correlated with percent body fat, age, and female sex. Gene expression of *CD68*, *CD11b*, and *CSF1R* but not Mphi was correlated negatively with glucose disposal rate but not after adjustment for percent body fat, age, and sex. However, adipose tissue expression of plasminogen activator inhibitor type-1 (PAI-1) and CD11 antigen-like family member C (CD11c), markers produced by macrophages, were negatively correlated with adjusted glucose disposal rate ($r = -0.28$, $P = 0.05$ and $r = -0.31$, $P = 0.03$).

CONCLUSIONS—ATMc is correlated with age and adiposity but not with insulin action independent of adiposity in healthy human subjects. However, PAI-1 and CD11c expression are independent predictors of insulin action, indicating a possible role for adipose tissue macrophage activation. ***Diabetes* 58: 385–393, 2009**

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Obesity is an inflammatory condition leading to chronic activation of an innate immune response (1). This inflammatory response has been implicated in the pathogenesis of obesity-associated complications, including atherosclerosis (2), nonalcoholic fatty liver disease (3), and insulin resistance (4). Adipose tissue is a primary site of obesity-induced inflammation and a complex organ containing adipocytes as well as connective tissue matrix, nerve tissue, stromal vascular cells, and immune cells. A cardinal feature of obesity-induced inflammation in adipose tissue is the recruitment of immune cells, specifically macrophages (5,6). Although the adipocyte is the defining cell of adipose tissue and does contribute to the production of inflammatory molecules (7), it appears that macrophages contribute substantially to the inflammatory signals that are induced by obesity (5,8–11).

Among the inflammatory factors whose expression is upregulated in adipose tissue with the onset of obesity, some have been implicated in recruitment of macrophages to adipose tissue, including chemokines, while others appear to be derived primarily from adipose tissue macrophages (ATMs). Studies in rodents indicate that ATMs are bone marrow–derived cells recruited to adipose tissue during periods of positive energy balance and increasing adiposity (5). However, the physiology of macrophage recruitment remains largely unknown. It has been hypothesized that a metabolic signal(s) or stress(es) leads to activation of endothelial cells, production of chemoattractants with subsequent transendothelial migration of monocytes (12), monocyte differentiation into mature macrophages, and ultimately macrophage activation. A few studies have also suggested that differentiation of adipocyte precursors into macrophage-like cells (6,13) can occur, although this remains controversial. Studies have implicated monocyte chemoattractant proteins, hypoxia, and angiogenesis in ATM recruitment. In particular, the adhesion molecule intercellular adhesion molecule 1 (ICAM1) is important in the recruitment of monocytes to sites of inflammation (14), its soluble plasma concentrations have been found to be positively associated with adiposity (15,16), and in previous microarray studies in mice adipose tissue *Icam1* expression was correlated with body mass (5).

Animal and human studies of obese and diabetic subjects indicate that adipose tissue macrophage content (ATMc) correlates with degree of adiposity (5,6,8,9,12). In a small, interventional study, the subcutaneous expression of CD68, a macrophage marker, correlated with insulin

resistance (10). In obese individuals, the degree of hepatic fibroinflammatory lesions or fat liver content is associated with omental or subcutaneous ATM infiltration (17–19). The association of ATMc with insulin resistance and nonalcoholic fatty liver disease indicates a role for ATMc in obesity-related complications. However, it is not clear yet whether ATMc or activation in healthy adults affects insulin action beyond their association with adiposity. In rodents, genetic manipulation of the activation of myeloid cells, including macrophages, alters insulin sensitivity (20–22).

In the present study, we examined in healthy nondiabetic individuals the association of subcutaneous ATMc and activation with direct measurements of both adiposity and whole-body insulin sensitivity. In addition, we investigated the relationship of ATMc and subcutaneous adipose tissue expression of genes potentially involved in attraction of macrophages into adipose tissue.

RESEARCH DESIGN AND METHODS

Adult Native Americans (at least one-half Pima or closely related Tohono O'odham Indians) participated in an ongoing longitudinal study to identify risk factors for type 2 diabetes and obesity. All subjects were nondiabetic, did not smoke or take medications at the time of the study, and were in good health, as determined by medical history, physical examination, and routine laboratory testing. Based on our database of previously studied volunteers, subjects were selected who had frozen subcutaneous adipose tissue in our tissue bank and measurements of both percent body fat and insulin action. For the study protocol, subjects were admitted to the Clinical Research Unit of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (Phoenix, AZ) for 8–15 days and were provided a standard weight-maintaining diet containing 50% of calories as carbohydrate, 30% as fat, and 20% as protein for at least 3 days before metabolic testing. The NIDDK Institutional Review Board and the Gila River Indian Tribal Council approved the study. Before participation, written informed consent was obtained.

Height was measured using a stadiometer; weight was obtained from an electronic digital scale. Percentage of body fat was measured by whole-body dual-energy X-ray absorptiometry. Glucose tolerance status was assessed by a 75-g oral glucose tolerance test (23). Insulin action was assessed at physiological insulin concentrations using a hyperinsulinemic-euglycemic glucose clamp (24). The mean steady-state insulin concentration during the clamps was 148 ± 37 pmol/l. The rate of total insulin-stimulated glucose disposal (*M*) was calculated for the last 40 min of insulin infusion and adjusted to steady-state glucose and insulin concentrations. All measurements derived from the glucose clamp were expressed per kilogram estimated metabolic body size (fat-free mass + 17.7 kg) (25). Plasma insulin concentrations were measured by two different radioimmunoassays used over time in our lab: Concept 4 (ICN, Costa Mesa, CA) and Access (Beckman Instruments).

Insulin assays. All measurements of insulin were normalized to original radioimmunoassay (a modified Herbert-Lau assay) using regression equations.

Fat biopsy, morphological analysis, and real-time quantitative PCR. After an overnight fast, subcutaneous adipose tissue is obtained by percutaneous needle biopsy of periumbilical fat depots using a 15-gauge needle through skin and anesthetized with 1% lidocaine. These tissue samples are immediately frozen in liquid nitrogen for subsequent studies, such as the one herein presented. Adipose tissue samples were fixed for 12–16 h at room temperature in zinc-formalin fixative (Anatech, Battle Creek, MI) and embedded in paraffin. Five-micrometer sections, cut at 50-μm intervals, were mounted on charged glass slides, deparaffinized in xylene, and stained for expression of CD68 as done previously (Caltag, Burlingame, CA) (5). For each individual tissue block, four different ×20 fields from each of five different sections were analyzed independently by two blinded evaluators. The total number of nuclei and the number of nuclei of CD68-expressing cells were counted for each field. The fraction of macrophage cells for each sample was calculated as the number of nuclei of CD68-expressing cells divided by the total number of nuclei in sections of the sample.

Total RNA was extracted from frozen adipose tissue (~50 mg) using a commercially available acid-phenol reagent (Trizol; Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Superscript III reverse transcriptase and random hexamer primers as described in the manufacturer's protocol (Invitrogen). Samples of cDNA were diluted 1:25 in nuclease-free water (Qiagen, Valencia, CA). PCR amplification mixtures (20 μl) contained 10 μl 2× PCR SYBR Green I Quantitect Master mix (Qiagen), 0.4 μl 25 μmol/l reverse and forward primer mix, and 11.6 μl diluted cDNA template. Real-time quantitative PCR was carried out using the DNA Engine Opticon (MJ Research, Waltham, MA) instruments with the following cycling parameters: polymerase activation, 15 min, 95°C; amplification for 40 cycles, 15 s, 94°C; 20 s, 58°C; 20 s, 72°C. After amplification, melting curve analysis was performed as described in the manufacturers' protocol (Qiagen). The control gene, casein kinase-1d (CSNK1d), was used to normalize simple load and reactions and for calculation of Δ-ΔC_t values, from which relative expression values were determined (arbitrary units [AU]). Human primer orientation and sequences were as follows: CSNK1d forward, 5′-AGGAGAAGAGGTTGCCAT CAAG-3′, and reverse, 5′-TCCATCACCATGACGTTGTAGTC-3′; CD68 forward, 5′-GCTACATGGCGGTGGAGTACAA-3′, and reverse, 5′-ATGATGAGAG GCAGCAAGATGG-3′; integrin αM (ITGAM)/CD11b forward, 5′-GAGTCCAAC GCTAATGTCAAGG-3′, and reverse, 5′-CCCGTAGAGAACAGCATCACAC-3′; CSF1R forward, 5′-GCTCAACCTCAAAGTCATGGTG-3′, and reverse, 5′-GAA GGTGTGCCTGTATGTGTCC-3′; LEP/Leptin forward, 5′-TGGCCCTATCTTTT CTATGTCC-3′, and reverse, 5′-GGTGACTTTCTGTTTGGAGGAG-3′; ICAM1 forward, 5′-CAGTCACCTATGGCAACGACTC-3′, and reverse, 5′-GCCTCACA CTTCACTGTCACCT-3′; CCL-2/MCP-1 forward, 5′-CAATCAATGCCCCAGTCA CC-3′, and reverse, 5′-GAATCCTGAACCCACTTCTGC-3′; plasminogen activator inhibitor type-1 (PAI-1) forward, 5′-CTGGTTCTGCCCAAGTTCTCC, and reverse 5′-CCACAAAGAGGAAGGGTCTGTC; hypoxia-inducible factor-1α (HIF-1a) forward, 5′-AGAACAAAACACACAGCGAAGC, and reverse, 5′-AATC AGCACCAAGCAGGTCATA-3′; vascular endothelial growth factor (VEGF) forward, 5′-AGCCTTGCCTTGCTGCTCTAC-3′, and reverse, 5′-ACCACTTCGT GATGATTCTGC-3′; C1QB forward, 5′-ACTGATGTTGCTCCTGCTCCTG-3′, and reverse, 5′-GGTCTCCCTTCTCTCCGAACTC-3′; S100A8 forward, 5′-GGC AAGTCCGTGGGCATC-3′, and reverse, 5′-ATCCAACTCTTTGAACCAGACG-3′; tumor necrosis factor (TNF) forward, 5′-TGCTTGTTCCTCAGCCTCTTC-3′, and reverse, 5′-GCTTGTCACTCGGGGTTCG-3′; matrix metalloproteinase-9 (MMP9) forward, 5′-ACCTTCTTGGCTACCTTTGACG-3′, and reverse, 5′-ATG GCTTTCCTTCTCCTCAGAC-3′; and CD11c forward, 5′-CAGAGATGCGTGGA GAGTCG-3′, and reverse, 5′-GACACCAAACTGGATGACGATG-3′.

Statistical analysis. Statistical analyses were performed using SAS software (SAS version 9.1; SAS Institute, Cary, NC). Non–normally distributed variables were log transformed ($\log_{10}$) to reduce skewness, and then normality was reevaluated. Unadjusted sex differences in variables under investigation were evaluated by Student's *t* tests or nonparametric (Kruskall-Wallis) test. Pearson or Spearman correlation analysis was used to quantify relationships between variables of interest before and after (partial correlations) adjustment for covariates. General linear regression models (GLMs) adjusted for age and body fat were used to investigate sex differences in variables of interest and to test for interaction terms. GLMs were used to test for nonlinear associations between ATMc and age both before and after adjusting for covariates. In these models, age was included as a quadratic term. Level of statistical significance was set at $P < 0.05$.

RESULTS

Anthropometric and metabolic characteristics and ATMc. Men were taller and heavier and had lower body fat than women. Women had higher 2-h plasma glucose concentrations than men. No differences between sexes in *M* were observed both before ($P = 0.2$) and after adjustment for age and body fat ($P = 0.5$). ATMc was assessed by immunohistochemical (IHC) analysis (Mphi) and gene expression of macrophage-specific genes (CD68, CSF1R, and CD11b or ITGAM). As previously reported, we found a higher expression of macrophage content in adipose tissue of women compared with men, which was statistically significant for *CSF1R* and *CD11b* (Table 1). However, after adjusting for adiposity, there were no sex-dependent differences in these markers ($P = 0.7$ and 0.3 for *CSF1R* and *CD11b*, respectively).

Measures of ATMc and their association with adiposity and age. No single measurement fully captures the heterogeneous ATMc of an adipose tissue sample. IHC analysis visually identifies cells but depends on the expression of a single protein or antigen, and given the heterogeneity of macrophage population in adipose tissue, no single antigen is likely to be expressed above the threshold of detection on all macrophages. Furthermore, ATMc is not uniform, and therefore, variations in macrophage

TABLE 1
Population characteristics

	Whole group	Men	Women
n	66	41	25
Age (years)	31 ± 8	31 ± 9	31 ± 6
Body weight (kg)	95 ± 22	99 ± 21*	89 ± 23
Height (cm)	167 ± 7	172 ± 6†	161 ± 4
BMI (kg/m^2)	34 ± 7	34 ± 7	35 ± 8
Body fat (%)	32 ± 7	29 ± 6†	38 ± 6
Fasting glucose (mg/dl)	88 ± 9	87 ± 8	89 ± 11
2-h glucose (mg/dl)	118 ± 29	112 ± 29‡	129 ± 28
M (mg · kg^{-1} · EMBS^{-1} · min^{-1})§	2.9 ± 1.2	3 ± 1.4	2.6 ± 0.8
Mphi (%)¶	27 ± 12	25 ± 11	30 ± 11
CD68 (AU)	1.07 ± 1.04	1 ± 1.15	1.18 ± 0.84
CSF1R (AU)	1.76 ± 1.73	1.3 ± 1.07†	2.52 ± 2.3
CD11b (AU)	0.09 ± 0.06	0.08 ± 0.06‡	0.1 ± 0.06
Leptin (AU)	34 ± 28	25 ± 20†	48 ± 33

Data are means ± SD. *$P = 0.08$, †$P < 0.01$, or ‡$P < 0.05$ for *t* test or Wilcoxon comparisons across groups. §*M* was only available in 65 (of 66) subjects (24 women). ¶Mphi was available only in 46 (of 66) (men/women: 31/15) individuals in which adipose tissue section, cell morphology, and staining met the required criteria. 2-h glucose, 2-h glucose after a 75-g glucose load; EMBS, estimated metabolic body size. AU, adipose tissue mRNA values were normalized using mRNA expression of csnk1d. CD68 (macrophage-myeloid–associated antigen), CSF1R (colony-stimulating factor-1 receptor), and CD11b (ITGAM).

content in fields analyzed may over- or underrepresent macrophage content in a given sample. The increase in ATMc was originally discovered when it was noted that the expression of macrophage-specific genes was correlated with adiposity in mice. Recognizing the limits of both approaches, we studied ATMc as assessed by IHC (fraction of CD68-expressing cells, Mphi) and gene expression of macrophage-specific genes. Mphi was correlated with gene expression of *CD68* ($r = 0.58$, $P < 0.0001$), *CSF1R* ($r = 0.46$, $P = 0.001$), and *CD11b* ($r = 0.43$, $P = 0.003$). *CD68* expression was correlated with *CSF1R* ($r = 0.68$) and *CD11b* ($r = 0.62$), and *CSF1R* and *CD11b* were correlated with each other ($r = 0.75$) (all $P < 0.0001$). These correlations suggest that each approach measures related and overlapping aspects of macrophage content, but none captures it entirely.

In this healthy population of young adults, subcutaneous ATMc as assessed by Mphi and macrophage gene expression was positively associated with body fat (shown in Fig. 1). ATMc was also associated with BMI ($r = 0.36$, $P = 0.02$, for Mphi; and $r = 0.34$, $r = 0.45$, $r = 0.49$, all $P < 0.01$, for *CD68*, *CSF1R*, and *CD11b* expression, respectively). This relationship between adiposity and ATMc did not differ by sex ($P > 0.3$ for both sex × body fat and sex × BMI interaction terms). Leptin expression, as a local measurement of adiposity, was also associated with Mphi ($r = 0.30$, $P = 0.04$) and expression of macrophage markers (*CD68*: $r = 0.26$, $P = 0.03$; *CSF1R*: $r = 0.23$, $P = 0.06$; *CD11b*: $r = 0.37$, $P = 0.002$).

No association was found between age and percent body fat ($P = 0.8$). However, as depicted in Fig. 2, ATMc increased with advancing age until ~31–33 years and then slightly decreased. Hence, a linear regression model of the relation between ATMc and age, including quadratic terms (age^2), was tested and showed that both linear (all $P <$

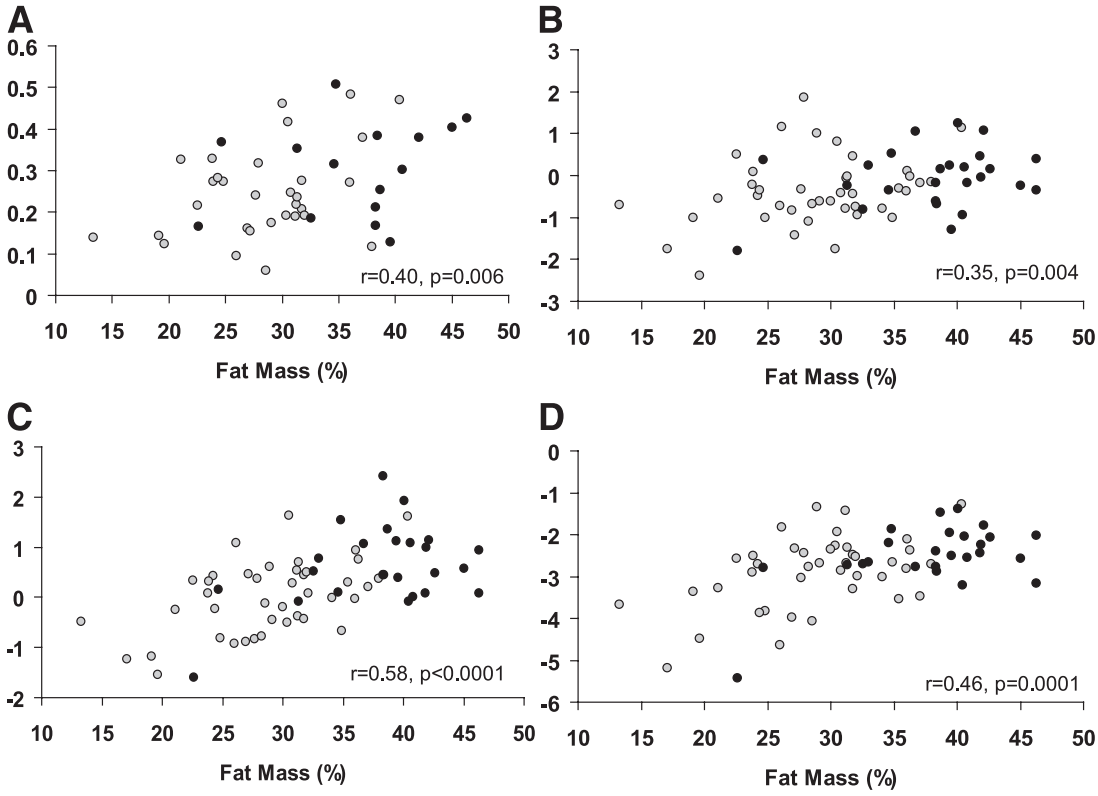

FIG. 1. Associations of adiposity with fraction of CD68$^+$ cells (*A*) and mRNA expression of macrophage markers (*B*, *CD68* expression [AU, ln]; *C*, *CSF1R* expression; *D*, *CD11b* expression). Simple correlation coefficients and *P* values for associations are given. ○, men; ●, women. Fraction of CD68$^+$ cells was available in 46 individuals (31 men and 15 women) in whom adipose tissue section, cell morphology, and staining met the required criteria. AU, mRNA values were normalized using mRNA expression of csnk1d.

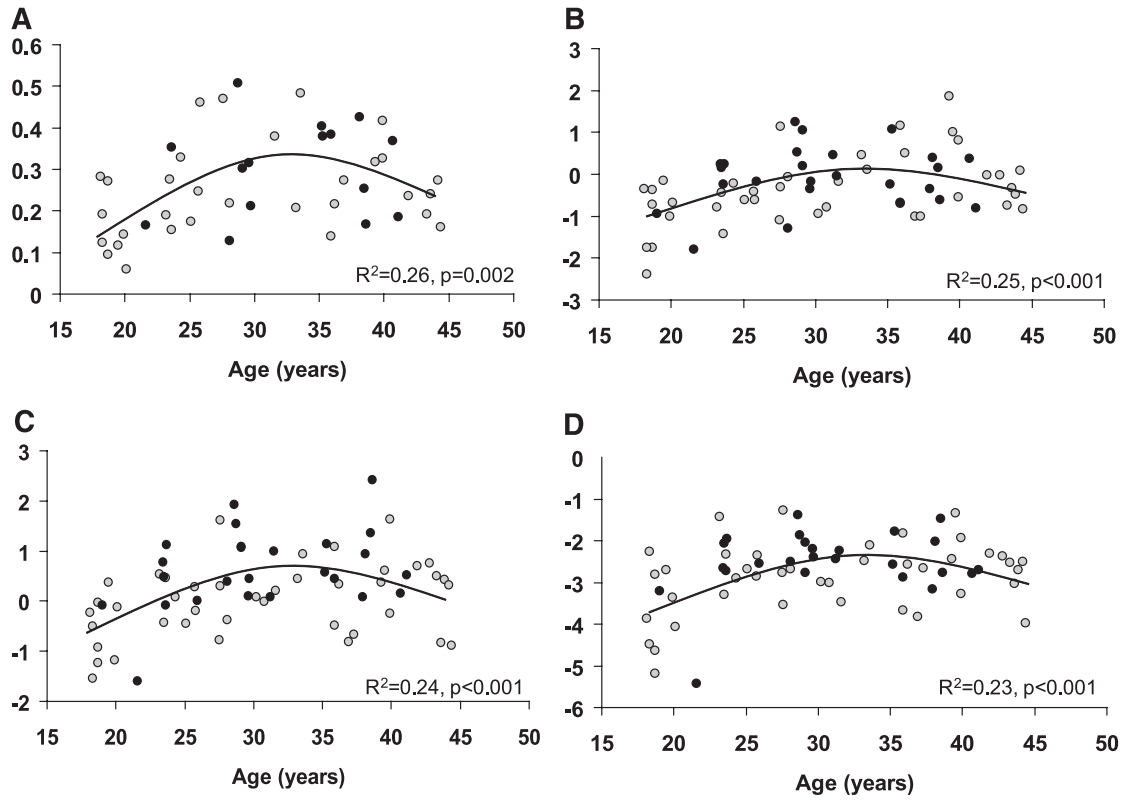

FIG. 2. Associations of age with fraction of CD68$^+$ cells (*A*) and mRNA expression of macrophage markers (*B*, *CD68* expression [AU, ln]; *C*, *CSF1R* expression; *D*, *CD11b* expression). Total r^2 and *P* values for the entire GLM are given. Models were built using linear and quadratic terms (macrophage content variable = age age^2). ○, men; ●, women. Fraction of CD68$^+$ cells was available in 46 individuals (31 men and 15 women) in whom adipose tissue section, cell morphology, and staining met the required criteria. AU, mRNA values were normalized using mRNA expression of csnk1d.

0.001) and quadratic (all $P < 0.01$) terms were statistically significant for all of the ATMc variables. Equations yielded by these models were as follows: Mphi (fraction of CD68$^+$ cells) = $-0.6 + 0.06 \times \text{age} - 0.001 \times \text{age}^2$; *CD68* expression (AU, ln) = $-5.6 + 0.34 \times \text{age} - 0.01 \times \text{age}^2$; *CSF1R* expression (AU, ln) = $-5.5 + 0.36 \times \text{age} - 0.01 \times \text{age}^2$; *CD11b* expression (AU, ln) = $-8.8 + 0.39 \times \text{age} - 0.01 \times \text{age}^2$.

Thus, in healthy, relatively young adults, macrophage content in a subcutaneous depot correlated with direct and indirect measures of adiposity and age independently. However, the fact that some of the macrophage markers were higher in women than men was dependent on differences in adiposity.

Association of ATMc with insulin action. As expected, body fat was negatively associated with *M* ($r = -0.34$, $P = 0.006$); a trend for an association between age and *M* was also noted ($r = -0.23$, $P = 0.07$). There was no association between Mphi and *M* (Fig. 3). Expression of CD68, CSF1R, and CD11b was negatively associated with *M* (Fig. 3). However, after adjustment for age and body fat, the negative correlation between macrophage gene expression and *M* was no longer significant (all $P > 0.4$). This relationship did not differ by sex ($P > 0.2$ for sex × ATMc [Mphi or markers] interaction terms in these models). These data argue that any systemic metabolic effect of subcutaneous ATMc that may exist cannot be separated from the dominant effects of adiposity.

Relationship between ATMc and inflammatory markers. We next studied the relationship between ATMc and the expression of inflammatory markers, including those implicated in recruitment and activation of macrophages. For this endeavor, only data from 56 individuals could be analyzed because of missing data for all of the inflammatory markers we measured or lack of enough adipose tissue to measure them in every subject from our initial study group ($n = 66$). We found that expression of ICAM1 was associated with percent body fat ($r = 0.33$, $P = 0.01$). Consistent with our murine data and a potential role for *Icam1* in ATM recruitment, expression of ICAM1 was also associated with ATMc as assessed by macrophage gene expression. The association of ATMc and ICAM1 expression is markedly attenuated when adjusted for percent body fat ($P = 0.03$, 0.08, and 0.12, for *CD68*, *CSF1R*, and *CD11b* expression, respectively), suggesting that any role of ICAM1 in ATM accumulation is not independent of adiposity (Table 2).

Chemokine monocyte chemoattractant protein-1 CCL-2 (also known as MCP-1) is a chemokine whose circulating blood concentrations are elevated in obese humans (26,27) and rodents (28) and whose expression is increased in adipose tissue of obese mice (28). In this study, CCL-2 expression correlates with body fat ($r = 0.29$, $P = 0.03$) and is also correlated with macrophage content, consistent with its role as chemoattractant for monocytes and macrophages. After adjustment for percentage of body fat, CCL-2 is still associated with CD68, CSF1R, and CD11b expression (all $P < 0.05$) but not with IHC-measured macrophage content ($P = 0.2$).

Macrophage migration inhibitory factor (MIF) is produced by both human mature adipocytes (29) and macrophages (30), is closely related with BMI (30), and is positively associated with insulin action (31). However, we

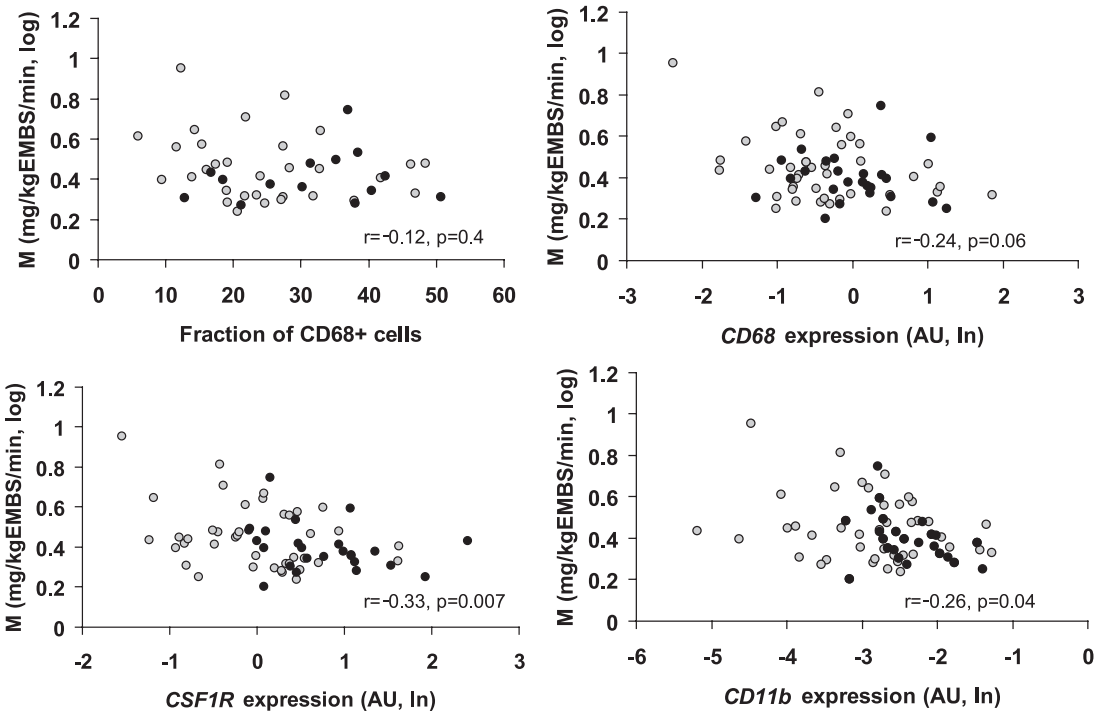

FIG. 3. Associations between insulin-stimulated glucose uptake and ATM content. Simple correlation coefficients and *P* values for associations are given. ○, men; ●, women. Fraction of CD68$^+$ cells was available in 46 individuals (31 men and 15 women) in whom adipose tissue section, cell morphology, and staining met the required criteria. EMBS, estimated metabolic body size. AU, mRNA values were normalized using mRNA expression of csnk1d.

did not observe any association between adipose tissue MIF expression and ATMc.

Adipocyte hypertrophy-associated hypoxia has been proposed to induce signals that recruit and retain macrophages in adipose tissue. HIF-1a is a master regulator of hypoxia-induced transcription and responses. Contrary to our predictions, expression in adipose tissue of HIF-1a and one of its downstream target genes, i.e., VEGF, correlate negatively with measures of macrophage content. No sex differences in adipose tissue expression of any of these genes were found before or after adjustment for age and body fat.

Adipose tissue macrophage activation relationship with fasting insulin and insulin action. Obesity not only increases ATMc but also increase macrophage inflammatory gene expression. We selected four inflammatory genes (TNF-α, PAI-1, complement factor C1qb [C1QB], and the Ca^{2+}-binding neutrophil cytosolic protein [S100a8]) that have been shown to be correlated with body mass and fasting plasma insulin concentrations and are substantially produced by ATMs in both animals (5) and humans (11). Expression of TNF-α and PAI-1 have previously been implicated in obesity-induced complications, and these factors are expressed by macrophages within the adipose tissue (5,11). C1QB is part of the classical complement system mostly expressed in the stroma fraction of the adipose tissue (32), and S100a8 is involved in immune and inflammatory processes (33). We also measured CD11 antigen-like family member C (CD11c), a marker of macrophage polarization toward a classically (proinflammatory) activated state (34), and MMP9, which is produced and secreted by activated ATMs (35). C1QB ($r = 0.33$, $P = 0.01$), TNF-α ($r = 0.51$, $P < 0.001$), S100a8 ($r = 0.30$, $P = 0.02$), CD11c ($r = 0.36$, $P = 0.007$), and MMP9 ($r = 0.43$, $P = 0.001$) expression, but not PAI-1, were associated with body fat. CD11c and MMP9 were also associated with age ($r = 0.38$ and 0.43, both $P < 0.01$). Sex differences were found for TNF-α, MMP9, and CD11c expression; however, these differences disappeared (all $P > 0.3$) after accounting for the differences in adiposity between sexes. TNF-α, PAI-1, MMP9, and CD11c were associated with both fasting insulin ($r = 0.37$, 0.37, 0.29, and 0.30, respectively, all $P < 0.05$) and M (Fig. 4; $r = -0.24$, $P = 0.08$; $r = -0.29$, $P = 0.03$; $r = -0.40$, $P < 0.01$; and $r = -0.40$, $P < 0.01$, respectively) and C1QB and S100a8 with fasting insulin ($r = 0.27$, $P = 0.05$; and $r = 0.23$, $P = 0.09$, respectively). After adjustment for percent body fat, the correlations of C1QB, TNF-α, and S100a8 with M or fasting insulin were no longer significant. However, after adjustment for age, sex, and percent body fat, the association between MMP9 and CD11c with fasting insulin was no longer significant ($P = 0.3$ and 0.09, respectively), and the association with M was attenuated although still significant for CD11c ($r = -0.25$, $P = 0.08$; and $r = -0.31$, $P = 0.03$, respectively). After adjustment for age, sex, and percent body fat, PAI-1 expression was still negatively correlated with M ($r = -0.28$, $P = 0.05$) and

TABLE 2
Associations between potential macrophage attractors and ATM content

	Mphi*	CD68	CSF1R	CD11b
CCL-2	0.32, 0.05	0.39, 0.003	0.39, 0.003	0.51, <0.001
ICAM	0.19, 0.3	0.39, 0.003	0.37, 0.005	0.34, 0.01
MIF	−0.21, 0.2	−0.05, 0.7	0.01, 0.9	0.15, 0.3
HIF-1	−0.40, 0.01	−0.23, 0.09	−0.21, 0.12	−0.12, 0.4
VEGF	−0.39, 0.02	−0.26, 0.05	−0.25, 0.07	−0.30, 0.02

Data are Spearman's correlation coefficients and *P* values for associations in 56 individuals. *$n = 38$ (men/women: 27/11) for correlations with Mphi.

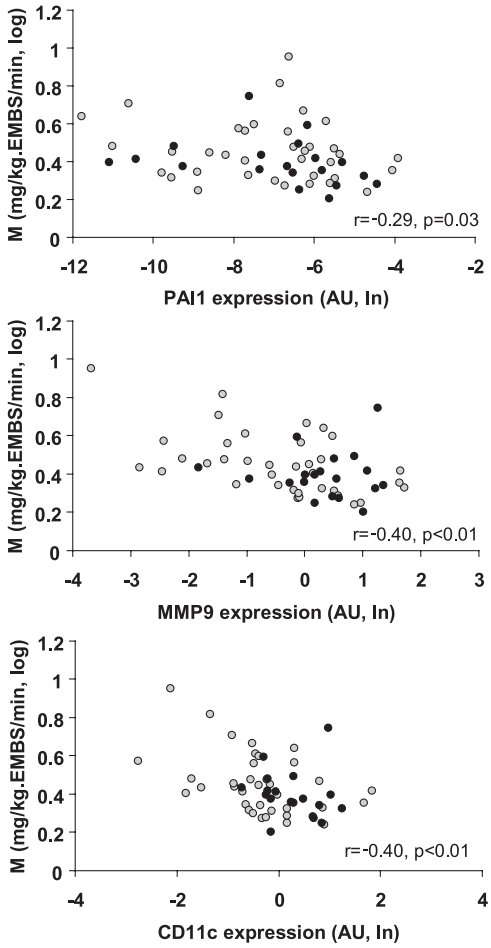

FIG. 4. Associations of adipose tissue expression of PAI-1, MMP9, and CD11c with insulin-mediated glucose uptake. Data are simple Spearman's correlation coefficients and *P* values for associations in 55 individuals (*M* was missed for one of the subjects). ○, men; ●, women.

positively correlated with fasting insulin ($r = 0.37$, $P = 0.01$).

DISCUSSION

In healthy, young subjects, adipose tissue macrophage content was associated with adiposity, adipose tissue leptin expression, and age but not with whole-body insulin–mediated glucose disposal after adjusting for adiposity. However, expression in adipose tissue of PAI-1, MMP9, and CD11c, putative markers of macrophage activation, was associated with whole-body insulin action. After adjustment for confounders, HIF1a and VEGF were negatively associated with subcutaneous adipose tissue macrophage content, as assessed by IHC analysis, and CCL-2 expression was positively associated with gene expression of macrophage markers (CD68, CSF1R, and CD11b). Expression of ICAM was associated with ATMc; however, this relationship was markedly attenuated after accounting for adiposity.

Subcutaneous and visceral ATMc increases with degree of adiposity (5,6,8,9,12,36,37), particularly intra-abdominal fat mass (37), and may have ethnic and metabolic determinants (37). Weight loss, either after bariatric surgery (8) or after diet and exercise (36), reduces adipose tissue macrophage content. Most (8,10,17,37), but not all (9), of previous human studies investigating this association have mainly studied Caucasian women. In this study, healthy male and female Pima Indians with a wide range of adiposity were included. No sex differences in ATMc were found when adiposity was accounted for, and no sex differences were observed for the association between adiposity and ATMc in agreement with a previous study (9). Subcutaneous ATMc was also positively associated with age in our population of young adults (18–44 years). These data indicate that at a certain age (31–33 years in our population), ATMc reaches a plateau, after which ATMc may decrease. Whether this is because of an issue of population selection (Pima Indians who develop diabetes at a young age would not be included, and so relatively lower-risk individuals would have been studied) cannot be determined. Associations of age and ATMc were described in studies of leptin-deficient (*Lep<ob/ob>*) mice in which clusters of macrophages were more prevalent as the obese mice aged (6) and in humans populations in which age was positively correlated with both subcutaneous (8,37) and omental (17,37) adipose tissue macrophage content. Hence, ATM accumulation is a common feature of increasing age and adiposity independent of sex, ethnicity, adipose tissue depot, and diagnosis of diabetes.

A number of in vitro and animals studies have implicated ATM recruitment and activation in the development of adverse metabolic consequences of obesity, in particular insulin resistance. Incubation of adipocytes in macrophage-conditioned medium activates an inflammatory and lipolytic transcriptional program (38,39) and decreases insulin-stimulated glucose uptake (38,40). Myeloid-specific deletion of IκB kinase β (20) reduces macrophage inflammation and protects from the detrimental effect of a high-fat diet on systemic insulin action, and conversely, myeloid-specific deletion of peroxisome proliferator–activated receptor γ increases the inflammatory profile of macrophages and reduces insulin sensitivity (21,22). An increase in adipose tissue expression of macrophage markers precedes the increase in fasting insulin in mice fed a high-fat diet during the development of obesity (6). Mice lacking the C-C motif chemokine receptor-2 (CCR-2) or Cbl-associated protein (Cap), which are both involved in migration of monocyte/macrophage cells, have lower macrophage content in adipose tissue and are partially protected from insulin resistance (41,42). Transgenic mice overexpressing CCL-2/MCP-1 showed increased macrophage accumulation in adipose tissue and were more insulin resistant than wild-type littermates (28,43).

Although these in vitro and animal studies indicate that adipose tissue macrophages may affect insulin action, evidence in humans is scarce. In obese subjects, omental ATMc was associated with severity of hepatic fibroinflammatory lesions but not with fasting insulin or the quantitative insulin sensitivity check index (QUICKI) after accounting for sex (17). Similar lack of association with insulin resistance was reported for subcutaneous ATMc in obese individuals undergoing bariatric surgery in whom changes (reduction) in infiltrating macrophages after weight loss were associated with changes in plasma levels of acute-phase proteins (serum amyloid and orosomucoid) but not with changes in fasting insulin or QUICKI (8). An association between insulin action (as measured by a tolbutamide-modified frequently sampled intravenous glucose tolerance test) and subcutaneous CD68 gene expression was found in 18 subjects, largely women, with normal glucose tolerance (10). However, this association was not adjusted for adiposity, a major confounder of this associ-

ation. In previous studies, insulin action was estimated using indirect methods. In the present study, using the euglycemic-hyperinsulinemic clamp technique, insulin action was weakly correlated with macrophage-specific gene expression (but not macrophage content as measured by IHC), but this association was no longer significant after adjustment for adiposity. It is possible that macrophage accumulation in other adipose depots, in particular in the visceral depot, may be associated with insulin action. However, a recent study reporting higher levels of macrophages in visceral compared with subcutaneous adipose tissue showed that the associations found between ATM counts and clinical parameters of obesity and its comorbid manifestations (including BMI, waist circumference, blood pressure, and fasting insulin) were of similar magnitude for both visceral and subcutaneous depots (37). In this study, we did not measure ATMc in visceral adipose tissue, but even assuming greater ATMc in visceral compared with subcutaneous adipose tissue, we do not know whether this would have also implied differences in the associations herein reported.

Macrophage activation, rather than number, may be more important in mediating the association between inflammation and lower insulin-mediated glucose uptake. TNF-α (a pleiotropic cytokine), PAI-1, S100a8 (33), C1QB (44), and MMP9 (45) are all proteins expressed at low levels in resting macrophages and upregulated by activation. Adipose tissue macrophages expressing the CD11c receptor are in a proinflammatory state (34), and activated ATMs produce and secrete MMP9 proportionally to the degree of adiposity (7,35,45). We found adipose tissue expression of TNF-α, S100a8, C1QB, CD11c, and MMP9 to be associated with adiposity in agreement with the hypothesis of obesity being an inflammatory condition. Furthermore, gene expression of PAI-1, MMP9, and CD11c was negatively associated with whole-body insulin sensitivity. The association between MMP9 was attenuated after adjusting for confounders. However, the association of CD11c and insulin action was still significant. PAI-1 was initially identified as an inhibitor to fibrinolysis but has inflammatory and metabolic function beyond its role in coagulation homeostasis. Insulin sensitivity is enhanced in PAI-$1^{-/-}$ mice on a high-fat diet compared with wild-type littermates (46). In humans, plasma PAI-1 activity is also negatively and independently associated with insulin sensitivity (47), and elevated baseline concentrations predict development of type 2 diabetes (48). It is important to acknowledge that within the adipose tissue, nonadipocyte cells, rather than adipocytes, are the primary source of PAI-1 (49,50) and that when irradiated PAI-$1^{+/+}$ mice are reconstituted with bone marrow from PAI-1–deficient mice, PAI-1 expression decreases by 66% (51). Taken together with our results, we can speculate that PAI-1 and other factors produced from activated adipose tissue macrophages might influence whole-body insulin action. In fact, another group has also reported that PAI-1 mRNA expression in abdominal subcutaneous adipose tissue is positively related with insulin resistance (52).

The molecular mechanisms responsible for ATM recruitment remain unknown. It has been reported that macrophage accumulation plays a major role in angiogenesis (53). Adipose tissue expansion, like tumors, is angiogenesis dependent (54). A hypoxic/vascular theory proposes that adipocytes may become hypoxic during a rapid expansion of adipose tissue and start secreting inflammatory cytokines (to increase blood flow) and angiogenic factors to promote formation of new blood vessels (55,56). Both leptin and CCL-2, whose expression in adipose tissue increases with adiposity, have angiogenic properties (57,58) and have been implicated in the recruitment of macrophages to adipose tissue (12,28,42,43). In this study, leptin and CCL-2 expression were positively related to ATMc. HIF-1 orchestrates the cellular responses to hypoxia. On activation, HIF-1a translocates to the nucleus and induces transcription of downstream target genes, such as VEGF, which is involved in angiogenesis. HIF-1 and VEGF are highly expressed by macrophages (59,60). VEGF is involved in angiogenesis during fat tissue formation (61,62), and this effect might be mediated by macrophages (63). In this study, expression of both HIF and VEGF mRNA was negatively associated with macrophage content. This negative association could represent adipose tissue failure to increase its vascularization in an attempt to limit further weight gain. However, this cannot be verified in this cross-sectional study.

Finally, we acknowledge that part of the interpretations of the results in this and other previous studies are based on correlations between gene expression data. Because of methodological issues (e.g., possible different amplification efficiency of samples), such correlations may overestimate (or rarely underestimate) true associations, although the fact that several ATM activation markers point to the same direction points to the reliability of the results.

In summary, our data indicate that aging and increasing adiposity are associated with adipose tissue macrophage infiltration. However, we found no independent association between subcutaneous ATMc and directly measured insulin action, indicating that ATMc per se is not a direct cause of insulin resistance. However, consistent with studies in mice, the expression of the macrophage activation marker PAI-1 was associated with insulin resistance, as was CD11c independent of adiposity. These data are consistent with a model in which macrophage activation rather than content is related to insulin action and suggest that further characterization of ATM populations and activation states may provide insights into the relationship between obesity-induced inflammation and insulin sensitivity.

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