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Clinical Research Article

Sex-specific Association Between Adipose Tissue Inflammation and Vascular and Metabolic Complications of Obesity

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Abstract

Context: Adipose tissue (AT) inflammation predisposes to insulin resistance and metabolic syndrome in obesity.

Objective: To investigate the association between adipocyte size, AT inflammation, systemic inflammation, and metabolic and atherosclerotic complications of obesity in a sex-specific manner.

Design: Cross-sectional cohort study.

Setting: University hospital in the Netherlands.

Participants: A total of 302 adult subjects with a body mass index (BMI) ≥ 27 kg/m^2.

Main outcome measures: We obtained subcutaneous abdominal fat biopsies and systematically assessed, in a sex-specific manner, associations of several parameters of AT inflammation (including adipocyte size, macrophage content, crown-like structures, and gene expression) to biomarkers of systemic inflammation, leukocyte number and function, and to the presence of metabolic syndrome, insulin resistance, and carotid atherosclerotic plaques, assessed with ultrasound.

Results: Adipocyte size was associated with metabolic syndrome and AT macrophage content with insulin resistance. In contrast, none of the AT parameters was associated with carotid atherosclerosis, although mRNA expression of the anti-inflammatory IL-37 was associated with a lower intima-media thickness. We revealed profound sex-specific differences, with an association between BMI and adipocyte size, and between adipocyte size and metabolic syndrome in men only. Also, only men showed an association between adipocyte size, AT expression of leptin and MCP-1, and AT macrophage numbers, and between AT inflammation (crown-like structure number) and several circulating inflammatory proteins, including high specificity C-reactive protein, and IL-6.

Conclusions: Inflammation in abdominal subcutaneous adipose tissue is more related to the metabolic than the atherosclerotic complications of obesity, and there are profound sex-specific differences in the association between BMI, adipocyte size, AT inflammation, and systemic inflammation, which are much stronger in men than women.

Key Words: obesity, inflammation, metabolic syndrome, atherosclerosis, macrophages

Abbreviations: AT, adipose tissue; BMI, body mass index; CLS, crown-like structure; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment for insulin resistance; IMT, intima media thickness; LDL-C, low-density lipoprotein cholesterol; MetS, metabolic syndrome; MRI, magnetic resonance imaging; PBMC, peripheral blood mononuclear cell; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; VLDL, very low-density lipoprotein.

The prevalence of obesity is rapidly rising worldwide. Obesity predisposes to insulin resistance and type 2 diabetes mellitus and is associated with an increased risk for atherosclerotic cardiovascular disease (1). Once obesity has developed, obtaining long-term weight loss is notoriously difficult. Hence, additional (pharmacological) strategies to prevent metabolic and cardiovascular complications of obesity are needed, and this requires elucidation of the mechanisms that link obesity to these complications.

Obesity is associated with the infiltration of adipose tissue (AT) by monocyte-derived macrophages that can induce AT inflammation, and this is critical for the development of

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insulin resistance and diabetes mellitus (2, 3). Expansion of AT volume can be due to an increase in adipocyte number or adipocyte volume. It is mainly adipocyte size that has been associated with the secretion of inflammatory cytokines and adipokines (4) and with insulin resistance (5). Also, the number of macrophages and crown-like structures (CLS; macrophages surrounding adipocytes) present in AT and AT expression of inflammatory cytokines such as TNF are associated with insulin resistance in small studies in selected patient groups (6–8).

Several important questions about the role of AT inflammation in the development of metabolic and vascular complications of obesity remain unanswered. First, the interactions between AT inflammation and systemic inflammation and circulating immune cells are largely unknown. Second, AT inflammation has been explored mainly in the context of metabolic complications of obesity and not in direct relation to the atherosclerotic cardiovascular complications. Finally, there is accumulating evidence that the role of inflammation in cardiometabolic disease is highly sex specific. We recently reported that among obese individuals, the presence of metabolic syndrome (MetS) was associated with circulating leptin and IL-6 concentrations in men, whereas in women this was inversely associated with the anti-inflammatory adipokine adiponectin (9). Therefore, it is essential to perform such analyses in a sex-specific manner.

In this study, we aimed to provide answers to these questions using a unique and deeply phenotyped cohort of 302 subjects with overweight or obesity. Approximately one-half of the subjects have MetS and one-half of them have atherosclerotic plaques in the carotid artery. We systematically assessed, in a sex-specific manner, associations of several parameters of abdominal subcutaneous AT inflammation (including adipocyte size, macrophage content, and gene expression) to biomarkers of systemic inflammation and leukocyte number and function, and the presence of metabolic and vascular complications of their obesity.

Methods

Study Population

The 300-Obesity cohort includes 302 individuals with a body mass index (BMI) >27 kg/m^2, enrolled at Radboud University Medical Center between 2014 and 2016. The cohort has been described in detail in previous publications (9–11), and the measurements performed are summarized in Fig. 1A. Lipid-lowering medication, if used, was interrupted for 4 weeks before blood collection to allow accurate assessment of MetS criteria and to prevent potential effects of statins on inflammatory parameters. Venous blood samples were obtained in the morning after an overnight fast. All participants gave written informed consent. This study was approved by the Ethical Committee of the Radboud University (Nr. 46846.091.13). Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Adipose Tissue Analysis

Abdominal fat volume and distributions (visceral vs subcutaneous adipose tissue [VAT vs SAT]) and liver fat content were assessed using magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy as described previously (10).

Additionally, we performed needle biopsies of abdominal subcutaneous adipose tissue after an overnight fast in 301 patients. The morphometry of individual fat cells was assessed using digital image analyses as described previously (12). The adipocyte cell diameters of all adipocytes in 4 microscopic fields of view were measured and expressed as area and Feretmin (Feret minimal diameter: the minimal diameter of each cell). To detect macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Serotec, Oxford, UK). The percentage of macrophages was expressed as the total number of macrophages divided by the total number of adipocytes counted in 15 random microscopic fields of view. A CLS was defined as an adipocyte surrounded by at least 3 macrophages (13).

For a small number of patients (n = 20, all female), we also performed biopsies from subcutaneous fat in the thigh region. cDNA libraries were prepared (iScript cDNA Synthesis Kit: Bio-Rad) using 1 μg total RNA isolated from these biopsies (Trizol reagent: Invitrogen). mRNA expression of cytokines and adipokines was assessed using real-time PCR and normalized to Ribosomal Protein L37a (RPL37A). The primers used in this process are listed in Table 1.

Cardiovascular Measurements

Carotid ultrasound was performed after an overnight fast or in the afternoon 6 hours after a standardized breakfast. In all circumstances, subjects abstained from consumption of caffeine, alcohol, vitamin supplements, or chocolate for 18 hours. Carotid plaque presence was defined as focal thickening of the wall at least 1.5× the mean carotid intima-medial thickness or a carotid intima-medial thickness greater than 1.5 mm (14). We used 3 measures for carotid plaques in our analysis: (1) the presence or absence of plaques; (2) the number of plaques; and (3) maximal plaque thickness.

Pulse wave analysis and pulse wave velocity were measured using AtCor SphygmoCor following the standard operating procedure.

Metabolic Parameters

Subjects were assessed for the presence of MetS, defined according to the National Cholesterol Education Program Adult Treatment Panel III criteria (15) as ≥3 features of:

- Abdominal obesity: waist circumference ≥102 cm in men or ≥88 cm in women.
- Triglycerides: serum TG ≥150 mg/dL (1.7 mmol/L) or treatment with lipid-lowering drugs.
- HDL cholesterol (HDL-C): serum HDL-C <40 mg/dL (1 mmol/L) in men or <50 mg/dL (1.3 mmol/L) in women.
- Blood pressure: ≥130/85 mm Hg or treatment for hypertension.
- Fasting plasma glucose: ≥100 mg/dL (5.6 mmol/L) or treatment for elevated blood glucose.

Additionally, serum insulin levels were measured, which in conjunction with glucose levels, allows the calculation of the insulin resistance using the homeostatic model assessment for insulin resistance (HOMA IR) (16).

Immune Cells

Immune cell counts were determined in fresh whole blood EDTA samples using the Sysmex XE-5000. Isolation of peripheral blood mononuclear cells (PBMCs) was performed as

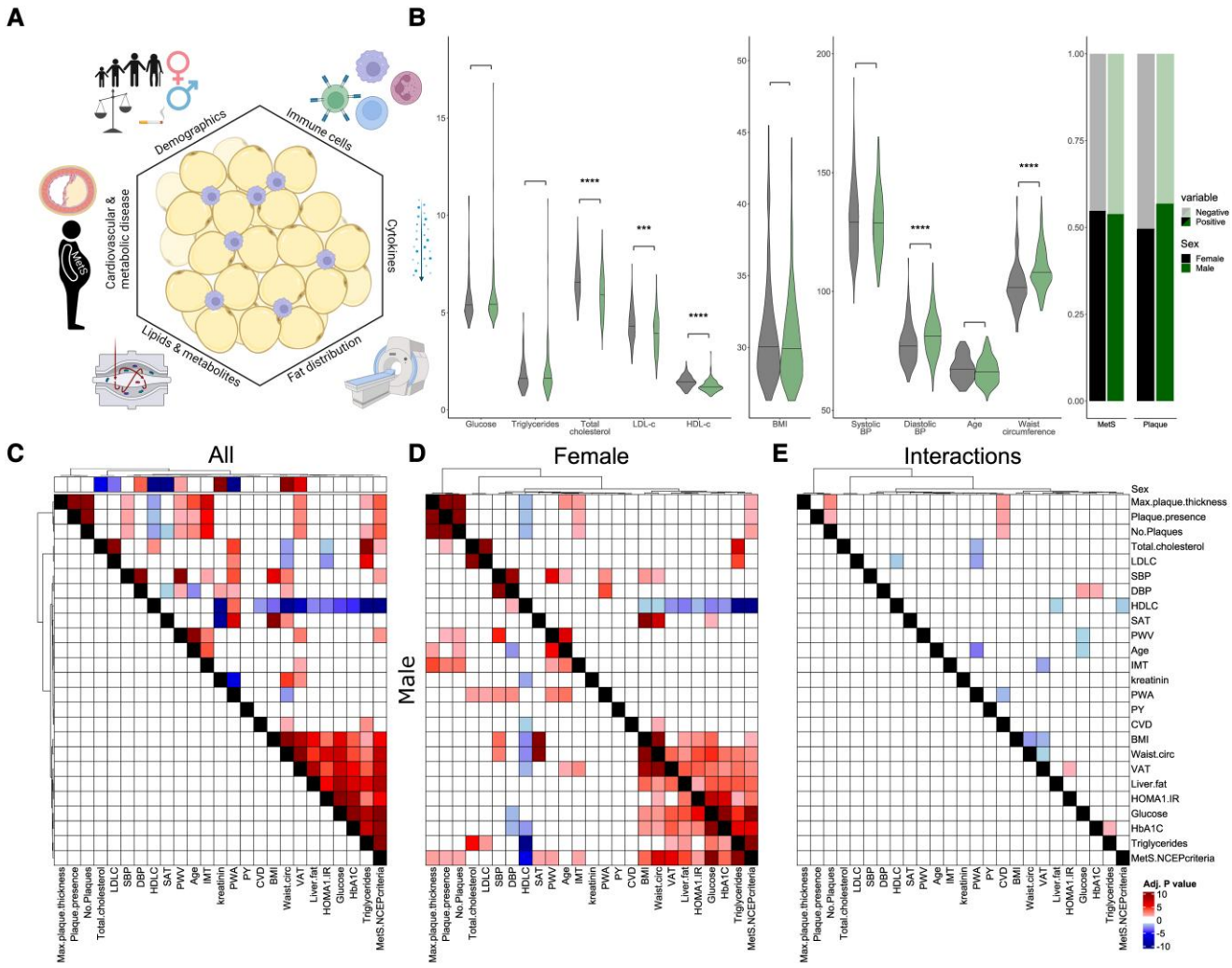

Figure 1. Clinical characteristics of the cohort. (A) Overview of the parameters investigated within the 300-Obesity cohort. (B) Baseline characteristics separated by sex (female [n = 135] in black/grey, male [n = 167] in green), *unadjusted *P* values calculated using Wilcoxon rank sum test, *** ≤ 0.001, **** ≤ 0.0001. (C-E) Significant correlations (Spearman) observed between the clinical parameters, the heatmaps represent the –log10 (*P* values) multiplied by the sign of the correlation coefficient. (C, D) Significance was determined using R's cor.test functionality and was adjusted for multiple testing using false discovery rate. (C) All patients combined. (D) Split for sex, correlations for women above the diagonal and men below. (E) Interaction effects measured by Fisher *Z* transformation to determine if correlations were significantly different between sexes, the color scale shows the sign of the z-transformation multiplied against the *P* value. For positive correlations, red indicates a stronger association with women and blue a stronger in men; this is inverted with negative associations. BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; PWA, pulse width amplitude; PWV, pulse width velocity; PY, pack years; SAT, subcutaneous adipose tissue; SBP, systolic blood pressure; VAT, visceral adipose tissue.

described previously (17). PBMCs were stimulated for 24 hours with various stimuli (lipopolysaccharide, Pam3Cys, *Candida albicans*, *Porphyromonas gingivalis*, *Staphylococcus aureus*, *Escherichia coli*, polyinosinic:polycytidylic acid, monosodium urate, C16). Supernatants were stored at −80°C and cytokines were measured with ELISA (IL-1β, IL-1Ra, IL-6, TNF).

Circulating Inflammatory Markers

Circulating inflammatory proteins (cytokines, chemokines, and adipokines) were measured in EDTA plasma. Using proximity extension assays technology, the levels of 176 inflammatory proteins from the Olink Cardiovascular II and Inflammatory panels were measured, as described before (18). In addition, ELISAs were performed for a selection of additional proteins, including AAT, adiponectin, high specificity C-reactive protein, IL-1β, IL-6, IL-18, IL-18BP, IL-38, leptin, resistin, and vascular endothelial growth factor, using commercial kits. Antibody Research Resource Identifiers are provided in Table 2.

Lipidomics

The Nightingale Biomarker Analysis Platform allowed the high throughput screening of 181 lipid and metabolite measures.

Statistical Analysis

All analyses were performed using the R programming language (scripts available on request). Because many measurements exhibiting nonnormal distributions cor.test() used Spearman's correlation, and all sample correlation *P* values were false discovery rate adjusted using the p.adjust() function in R, adjusted values ≤.05 were considered significant.

Table 1. Primers used for assessing cytokine/adipokine expression in adipose tissue

Gene	Forward primer	Reverse primer
Adiponectin	ATCGGTGAAACCGGAGTACC	GCATGTTGGGGATAGTAACGTAA
CD68	GCTGGCTGTGCTTTTCTCG	GTCACCGTGAAGGATGGCA
CD68(2)	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
IL-18	GGCCTCTATTTGAAGATATGACTGATT	CCTCTAGGCTGGCTATCTTTATACATACT
IL-18bp	ATGAGACACAACTGGACACCA	GCCAGGTCACTTCCAATGC
IL-18Rβ	CCACAGTTACTTGGAGAGGCTTAAA	GGCATGTGGTAGCGCATTT
IL-1α	ATCATGTAAGCTATGGCCCACT	CTTCCCGTTGGTTGCTACTAC
IL-1Ra	GCCTCCGCAGTCACCTAAT	TCCCAGATTCTGAAGGCTTG
IL-37	CAGCCTCTGCGGAGAAAGGAAGT	GTTTCTCCTTCTTCAGCTGAAGGGATGGAT
Leptin	GGTTGCAAGGCCCAAGAA	ACATAGAAAAGATAGGGCCAAAGC
MCP-1	CCAGTCACCTGCTGTTATAAC	TGGAATCCTGAACCCACTTCT
RPL37A	TAATACGACTCACTATAGGCTTTCTGGGCTC	TCTTCATGCAGGAACCACAG
TNF	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC

Forward and reverse sequences of primers used for RT-PCR to study mRNA expression levels.

Table 2. ELISA kits and antibodies used in histology, with manufacturer ID and RRID

Description	Manufacturer	Manufacturer ID	Antibody registry
Human IL-1β ELISA kit	R&D Systems	DY201	AB_2848158
Human IL-6 ELISA kit	Sanquin	M9316	AB_10851499
Human TNF-α ELISA kit	R&D Systems	DY210	AB_2848160
Human IL-22 ELISA kit	R&D Systems	DY782	AB_2928043
Human IL-17 ELISA kit	R&D Systems	DY317	AB_2928042
Human IFNγ ELISA kit	Sanquin	M9333	AB_2934300
Human IL-1Ra ELISA kit	R&D Systems	DRA00B	AB_2916104
Human Resistin ELISA kit	R&D Systems	DY1359	AB_2893494
Human Leptin ELISA kit	R&D Systems	DY398	AB_2861156
Human Adiponectin ELISA kit	R&D Systems	DY1065	AB_2861158
Human AAT ELISA kit	R&D Systems	DY1268	AB_2934301
Human IL-1Ra ELISA kit	R&D Systems	DY280	AB_2934302
Human IL-18BP ELISA kit	R&D Systems	DBP180	AB_2934303
Human hsCRP ELISA kit (Plasma)	R&D Systems	DY1707	AB_2928088
IL38	R&D Systems	DY9110	AB_2934304
CD68 AB for AT staining	BioRad	MCA1815	AB_322866
Human IL-18 ELISA kit	Simple plex (Biotechne/R&D)	SPCKB-PS-000501	Multi-analyte cartridges no antibody registry ID
Human IL-6 ELISA kit (Plasma)	Simple plex (Biotechne/R&D)	SPCKB-PS-000190	
Human VEGF ELISA kit (Plasma)	Simple plex (Biotechne/R&D)	SPCKB-PS-000330	
Human IL-1b (plasma)	Simple plex (Biotechne/R&D)	SPCKB-PS-000216	

For testing associations after correcting for other factors (eg, BMI), residuals from linear models were correlated to assess if relationship exists when accounting for these potential confounding issues. Fisher Z transformation was used to compare correlation coefficients between sexes (women being r_1 and men r_2; therefore, positive values had stronger correlation in women and vice versa).

$$Z_{obs} = \frac{(\text{atanh}(r_1) - \text{atanh}(r_2))}{\sqrt{\frac{1}{N_1 - 3} + \frac{1}{N_2 - 3}}}$$

All correlation results indicate the aspect of the cohort tested (R_s = all, R_{sf} = female, R_{sm} = male), degrees of freedom (in brackets), Spearman Rho, and adjusted P value (unless indicated otherwise).

All analyses were performed using "R" programming language (R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinical Characteristics of the Cohort

Clinical characteristics of this cohort have been published previously (9, 11, 19). Briefly, approximately 50% of the 302 participants fulfilled the criteria of MetS. Also, in approximately 50% of the subjects, atherosclerotic plaques were

present in the carotid artery (Fig. 1B). In addition to a strong positive association between MetS and its individual components, there is also a clear correlation between the metabolic and atherosclerotic complications of obesity: the presence of MetS shows strong positive associations with all measures of carotid plaques: plaque presence (r_s (298) = .21, P < .001), number of plaques (r_s (298) = .24, P < .001), and maximal plaque thickness (r_s (298) = .24, P < .001), underscoring the importance of MetS in the development of atherosclerosis in obese individuals.

MetS also demonstrates associations with other aspects of metabolic dysfunction: insulin resistance (r_s (297) = .28, P < .001), HbA1c (r_s (296) = .37, P < .001), and liver fat (measured with MRI) (r_s (268) = .37, P < .001). The presence of carotid plaques was significantly associated with VAT volume (r_s (274) = .2, P = .026), age (r_s (298) = .15, P = .025), HDL-C (r_s (296) = −.16, P = .02), and systolic blood pressure (r_s (298) = .16, P = .014), but not with low-density lipoprotein cholesterol (LDL-C; r_s (291) = .09, P = .24).

The significant association between MetS and plaques remained present in both men and women analyzed separately. Differential association between the different fat deposit volumes and sex were observed, as previously published, with a strong correlation between BMI and VAT volume in men (r_{sf} (119) = .23, P = .05; r_{sm} (151) = .49, P < .001; z = −2.56, P = .011), but no correlation in women. Expanding on this difference in fat deposits, VAT volume was associated with carotid intima media thickness (IMT) only in men (r_{sf} (116) = −.06, P = .77; r_{sm} (151) = .28, P < .001; z = −2.78, P = .006).

Inflammatory Characteristics of Subcutaneous Adipose Tissue

Comparison of abdominal and thigh SAT

In addition to adipocyte size, we assessed various parameters of AT inflammation, including the number of macrophages and CLS and the expression of various pro- and anti-inflammatory cytokines, chemokines, and adipokines.

To first explore potential differences between abdominal and thigh SAT composition, we compared the previously mentioned parameters between these 2 anatomical locations from the 20 female participants who underwent these paired biopsies (Fig. 2). To assess potential differences between biopsy site, principal component analysis was used to identify potential clustering of the samples. The first 2 principal components addressed 70% of the variation observed within the entirety of the data, and, as shown in Fig. 2, there was no separation of the biopsy sites, also evidenced by the lack of significant correlations between these components and biopsy site. To reinforce this conclusion, only the mRNA expression of IL-1Ra and IL-18 were significantly different between biopsy sites.

All analyses presented in the rest of this article relate to the abdominal AT biopsies that were performed in the 301 study participants.

Abdominal adipose tissue morphology and inflammation

Unsupervised clustering reveals a strong correlation of the mRNA expression of various inflammatory proteins with each other (CD68, TNF, IL-1A, IL-18Rß, and IL-37), which all have highly significant Spearman rho values greater than 0.85 (Fig. 3A and B).

Adipose tissue inflammation is strongly driven by macrophage infiltration. This is quantified by the number and percentage of CD68-positive cells/field and by the number of CLS/field, which are all strongly associated. The role of adipocyte size in AT inflammation is highlighted by the significant positive correlations of adipocyte size (mean area) with the expression of MCP-1 (r_s (248) = .21, P = .003) and with the percentage of macrophages (r_s (259) = .15 P = .026) and CLS (r_s (259) = .17, P = .011). Minimal adipocyte diameter (Feretmin) showed inverse correlations with expression of the anti-inflammatory adiponectin (r_s (252) = −.17, P = .017), IL-18Rß (r_s (252) = −.15, P = .037), and IL-37 (r_s (252) = −.14, P = .04).

Importantly, all 3 measures of macrophage infiltration have strong associations with the gene expression levels of MCP-1 (r_s for number of CD68 cells: (265) = .23, P < .001), *IL-18* (r_s (231) = .17, P = .024), and leptin (r_s (269) = .19, P = .004). This association highlights the importance of MCP-1 in attracting circulating monocytes, and MCP-1 expression is not only associated with the numbers of AT macrophages, but also with the presence of CLS (r_s (265) = .22, P < .001). The number of CLSs, but not number of macrophages per se, was inversely associated with the expression of the anti-inflammatory mediators IL-37 and adiponectin (r_s (269) = −.2, P = .002). Of particular interest is IL-37, which is regarded as an atheroprotective cytokine, whose expression demonstrates a negative correlation with MCP-1 expression (r_s (279) = −.14, P = .033) in addition to the presence of CLS (r_s (269) = −.13, P = .048), but not the presence of macrophages.

Sex-dependent differences in AT inflammation

Examining the correlations in men and women separately reveals several important observations. First, regarding the importance of adipocyte size in AT inflammation, the adipocyte size (area mean) was associated with the number of macrophages (r_{sf} (116) = −.17, P = .11; r_{sm} (139) = .11, P = .3; z = −2.27, P = .002; Fig 3B, C, and E), percentage of macrophages (r_{sf} (116) = .03, P = .81; r_{sm} (139) = .24, P = .01; z = −1.74, P = .008), and the expression of MCP-1 (r_{sf} (115) = .13, P = .22; r_{sm} (129) = .26, P = .01; z = −1.03, P = .3) only in men. Second, adipocyte size is significantly correlated with leptin expression only in men (R_{sf} (115) = .05, P = .67; r_{sm} (133) = .22, P = .03; z = −1.36, P = .18) and IL-18 (r_{sf} (97) = .16, P = .18; r_{sm} (113) = .25, P = .02; z = −0.7, P = .48), although these parameters were not significantly different between sexes. Third, in men only, the number of CLSs was significantly associated with the expression of MCP-1 (r_{sf} (123) = .08, P = .43; r_{sm} (137) = .37, P < .001; z = −2.19, P = .03; Fig 3E), as well as IL-18 (r_{sf} (105) = .13, P = .25; r_{sm} (122) = .33, P < .001; z = −1.62, P = .11). Meanwhile, in women, the expression of IL-18BP was associated with the numbers of macrophages (r_{sf} (77) = .27, P = .04; r_{sm} (52) = .1, P = .56; z = 0.99, P = .32), and leptin (r_{sf} (110) = .33, P < .001; r_{sm} (130) = .27, P = .001; z = 0.58, P = .56).

A further interesting observation is the importance of AT IL-18 expression in women, which uniquely shows a negative association with a number of inflammatory markers; TNF (r_{sf} (110) = −.29, P = .006; r_{sm} (130) = −.04, P = .75; z = −2.02, P = .04), IL-1a (r_{sf} (110) = −.27, P = .012; r_{sm} (130) = −.02, P = .86; z = −1.96, P = .05), IL-18Rb (r_{sf} (110) = −.29, P = .006;

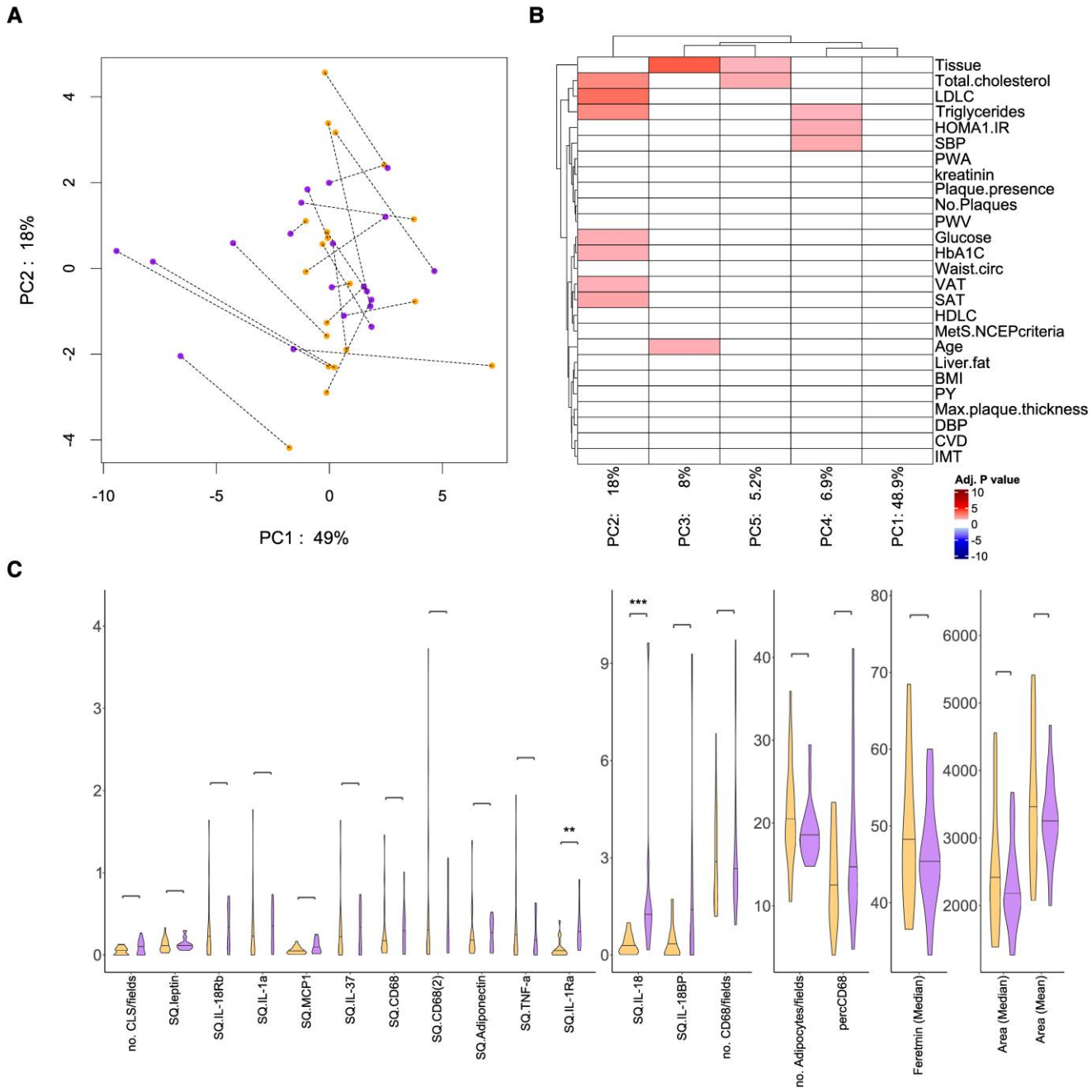

Figure 2. Adipose tissue parameters in thigh and abdominal fat biopsies. To compare biopsy location site, paired subcutaneous biopsies were obtained from both the abdomen and thigh in 20 female patients. (A) Principal component analysis showing the first 2 components that explain approximately 70% of the variance. No separation of the abdominal samples (orange) and thigh samples (purple) was observed (lines indicate the paired sample). (B) Heatmap correlating the principal components to the individual markers measured; only PC3 and PC5 show a significant correlation to tissue. (C) Plotting the individual adipose tissue parameters measured; only IL-1Ra and IL-18 expression showed any significant difference between tissue biopsy site. *P value calculated using Wilcoxon rank sum test, ** ≤ 0.01, *** ≤ 0.001.

r_{sm} (130) = 0, P = .99; z = −2.28, P = .02), and IL-37 (r_{sf} (110) = −.27, P = .011; r_{sm} (130) = −.02, P = .9; z = −2.03, P = .04).

Association Between AT Inflammation and Metabolic and Atherosclerotic Complications of Obesity

To explore how the AT could affect the metabolic and vascular complications of obesity, we next calculated the associations between all AT parameters and clinical parameters related to MetS and atherosclerosis.

Adipocyte size (expressed as area) is strongly associated with BMI (r_s (260) = .25, P = .0045; Fig. 4A) and SAT volume (r_s (247) = .31, P < .001), and this is only because of an association in men and not in women (SAT: r_{sf} (108) = .006, P = .83; r_{sm} (135) = .29, P = .004; z = −2.39, P = .019); BMI: r_{sf} (116) = .1, P = .7; r_{sm} (140) = .38, P < 0.001; z = −2.39, P = .017).

SAT volume also has significant associations with the mRNA expression of leptin (r_s (268) = .29, P < .001), CD68 (r_s (268) = .19, P = .036), IL-37 (r_s (268) = .19, P = .048),

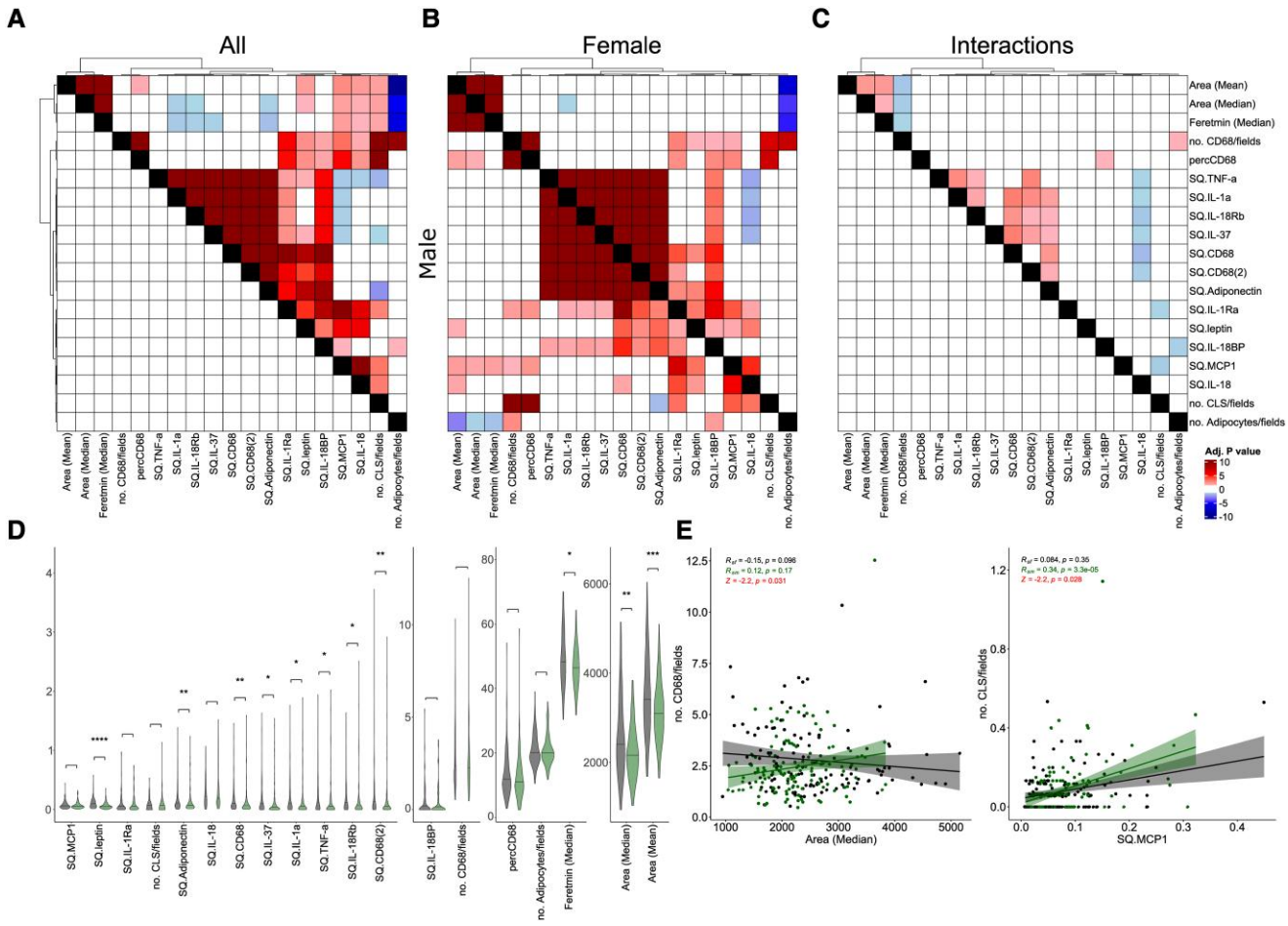

Figure 3. Inflammatory characteristics of subcutaneous adipose tissue biopsies. (A, B) Significant correlations (Spearman) observed between the adipose tissue parameters, the heatmaps represent the –log10 (*P* values) multiplied by the sign of the correlation coefficient. Significance was determined using R's cor.test functionality and was adjusted for multiple testing using false discovery rate. (A) All patients combined. (B) Split for sex, correlations for women above the diagonal and men below. (C) Interaction effects measured by Fisher *Z* transformation to determine if correlations were significantly different between sexes. The color scale shows the sign of the *z* transformation multiplied against the *P* value; for positive correlations, red indicates a stronger association with women and blue a stronger in men. This is inverted with negative associations. (D) Baseline characteristics of the adipose tissue parameters measured. *Unadjusted *P* values calculated using Wilcoxon rank sum test, * ≤ 0.05, ** ≤ 0.01, **** ≤ 0.0001. (E) Diametrically opposed correlations exhibited between sexes. Plots indicate Spearman rho and uncorrected *P* values women and men (black/grey and green, respectively), in addition to Fisher *Z* transformation (in red). Adipocyte size is shown as area (mean and median) and as minimal diameter measured (Feretmin), as well as number of adipocytes/field. Macrophage infiltration was characterized by number of CD68+ cells measured, as a percentage of total cells, and if they were organized into a crown-like structure (CLS). Inflammatory markers were assessed via RT-quantitative PCR, and the starting quantities (SQ) of all markers measured were normalized to the reference gene RPL37A.

and IL-1α (r_s (268) = .18, P = .049); this was not different between men and women.

Regarding the metabolic complications of obesity, we report significant correlations between:

1. Adipocyte size (expressed as mean area) and the presence of MetS (r_s (260) = .2, P = .036), only in men and not in women.
2. The percentage of macrophages present in AT is associated with insulin sensitivity (HOMA1.IR: r_s (277) = .19, P = .036) and HbA1C (r_s (276) = .20, P = .03) in the entire cohort.
3. The amount of liver fat and the AT mRNA expression of IL-18 (r_s (223) = .29, P = .011), MCP-1 (r_s (253) = .23, P = .0014), and IL-1Ra (r_s (221) = .22, P = .036), in the entire cohort.

To investigate potential relations between AT inflammation and atherosclerotic plaque development, we calculated the association between the AT parameters and the 3 carotid artery plaque parameters. Overall, we observed no significant associations with the various plaque parameters. The IMT is generally regarded as a measure of preclinical atherosclerosis, and we observed negative associations in the entire cohort between IMT and the AT mRNA expression of the anti-inflammatory proteins IL-1α (r_s (279) = –.19, P = .036), adiponectin (r_s (279) = –.19, P = .0.36), and IL-37 (r_s (279) = –.18, P = .049).

Another remarkable sex-specific association was observed between MCP-1 expression and total cholesterol (r_{sf} (128) = –.03, P = .91; r_{sm} (145) = .32, P = .01; z = –2.96, P = .003), and LDL-C (r_{sf} (126) = –.06, P = .83; r_{sm} (142) = .3, P = .02; z = –3.06, P = .0022), with only a strong correlation in men, but not in women (Fig 4D).

Association Between AT Inflammation and Circulating Lipoprotein Parameters

As shown in Fig. 4, there was a clear sex-specific association between expression levels of MCP-1 and IL-1Ra in AT and

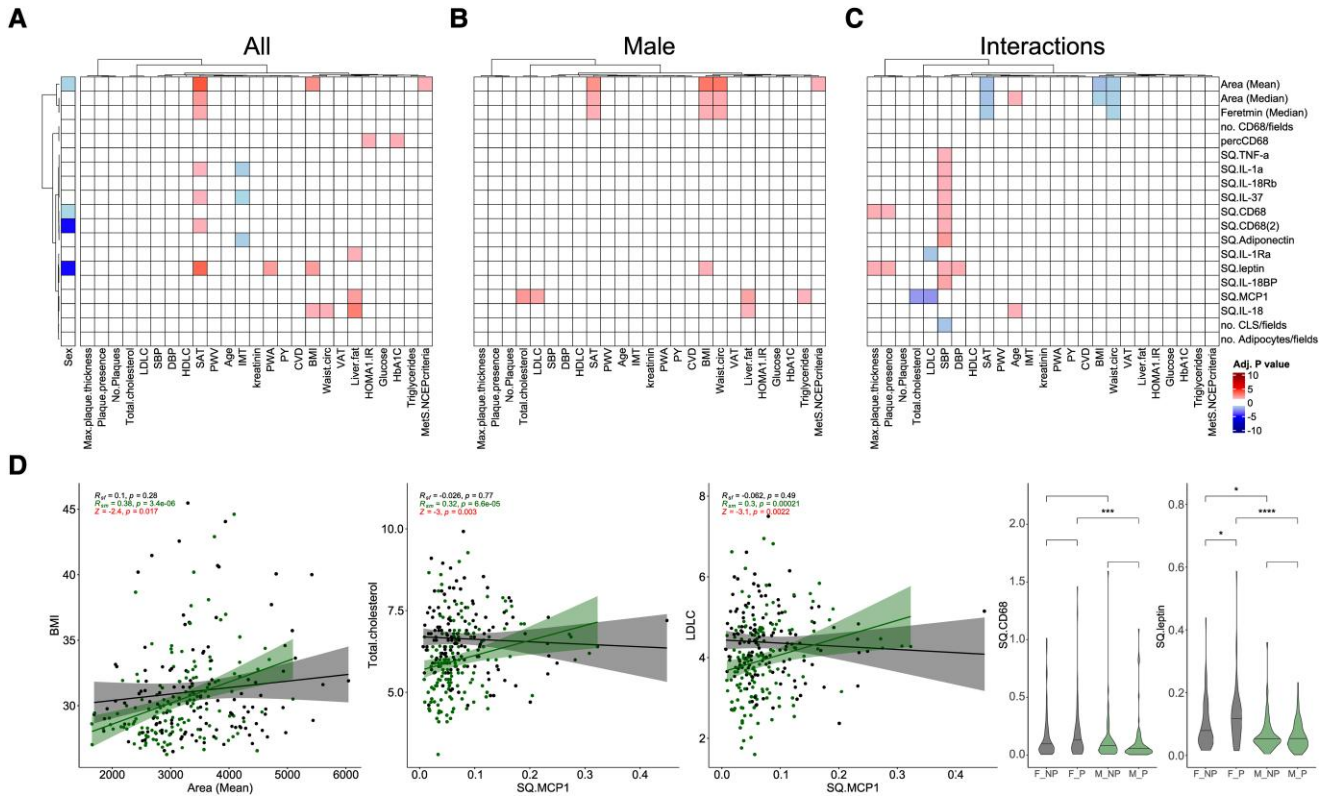

Figure 4. Association between AT inflammation and cardiometabolic and cardiovascular complications of obesity. Significant correlations (Spearman) observed between the adipose tissue parameters and clinical characteristics of the cohort; the heatmaps represents the –log10 (*P* values) multiplied by the sign of the correlation coefficient. Significance was determined using R's cor.test functionality and was adjusted for multiple testing using false discovery rate. (A) All patients combined. (B) Male patients only. (C) Interaction effects measured by Fisher *Z* transformation to determine if correlations were significantly different between sexes. Plots to illustrate the differences in associations observed between sexes; the color scale shows the sign of the *z* transformation multiplied against the *P* value, for positive correlations, red indicates a stronger association with women and blue a stronger in men. This is inverted with negative associations. (D) Scatterplots show the Spearman correlation values for each sex (women in black/grey; men in green); in red is the Fisher *Z* transformation to highlight differences between these. Violin plots highlight the pairwise comparisons between patients with and without a plaque and between sexes. *Unadjusted *P* values calculated using Wilcoxon rank sum test, * ≤ 0.05, *** ≤ 0.001, **** ≤ 0.0001.

circulating total and LDL-C, with strong a strong positive association in men only. To explore in more detail how AT inflammation relates to circulating lipoproteins, we made use of the lipoprotein parameters that were measured using the Nightingale lipidomics platform.

Of the 181 lipid and metabolite species, 86 (48%) had a significant positive association with adipocyte size after multiple testing correction. This association remained for almost all of these species following BMI correction (77 species), and most of these associations were not sex specific. Because of the reduced power arising from smaller sample sizes when stratifying the sexes, the majority of these associations was not observed despite similar correlation coefficients. However, 1 sex-specific association observed was AT expression of MCP-1, which demonstrated a clear difference between men and women in the Fisher *Z* transformation plot (Fig 5B). Checking these correlation coefficients shows that the significant associations observed in Fig. 5A is driven entirely by the male patients because these generally showed an inverse relationship between a strong positive male correlation and a weak female correlation.

We subsequently focused on the AT macrophage infiltration and its major driver MCP-1 expression. The number of macrophages and CLSs were not associated with lipoprotein-related parameters, but there were significant associations between phenylalanine (R_s (275) = .19, P = .022; R_{sf} (123) = .17, P = .34; R_{sm} (148) = .21, P = .17; z = –0.33, P = .74) and glycoprotein acetyls (R_s (277) = .18, P = .034; R_{sf} (123) = .03, P = .91; R_{sm} (150) = .29, P = .039; z = –2.17, P = .03) with CLS; and between glutamine (R_s (277) = .17, P = .044; R_{sf} (123) = .12, P = .53; R_{sm} (150) = .22, P = .14; z = –0.84, P = .4) and glucose (R_s (267) = .19, P = .025; R_{sf} (118) = .28, P = .15; R_{sm} (147) = .1, P = .57; z = 1.56, P = .12) with percentage macrophages.

For MCP-1 expression, there was a clear association with many parameters of very low LDL (VLDL) and LDL lipoproteins, for example, triglycerides in VLDL (R_s (278) = .17, P = .048; R_{sf} (127) = .11, P = .54; R_{sm} (147) = .23, P = .13; z = –0.97, P = .13) or LDL (LDL-triglycerides: R_s (278) = .2, P = .014; R_{sf} (127) = .03, P = .91; R_{sm} (147) = .31, P = .03; z = –2.41, P = .016).

Association Between AT Inflammation With Circulating Leukocyte Numbers and Cytokine Production Capacity

To explore potential mechanisms of how AT inflammation affects the development of insulin resistance and MetS and atherosclerosis formation, we carefully explored associations between AT parameters and circulating leukocytes and PBMC cytokine production capacity.

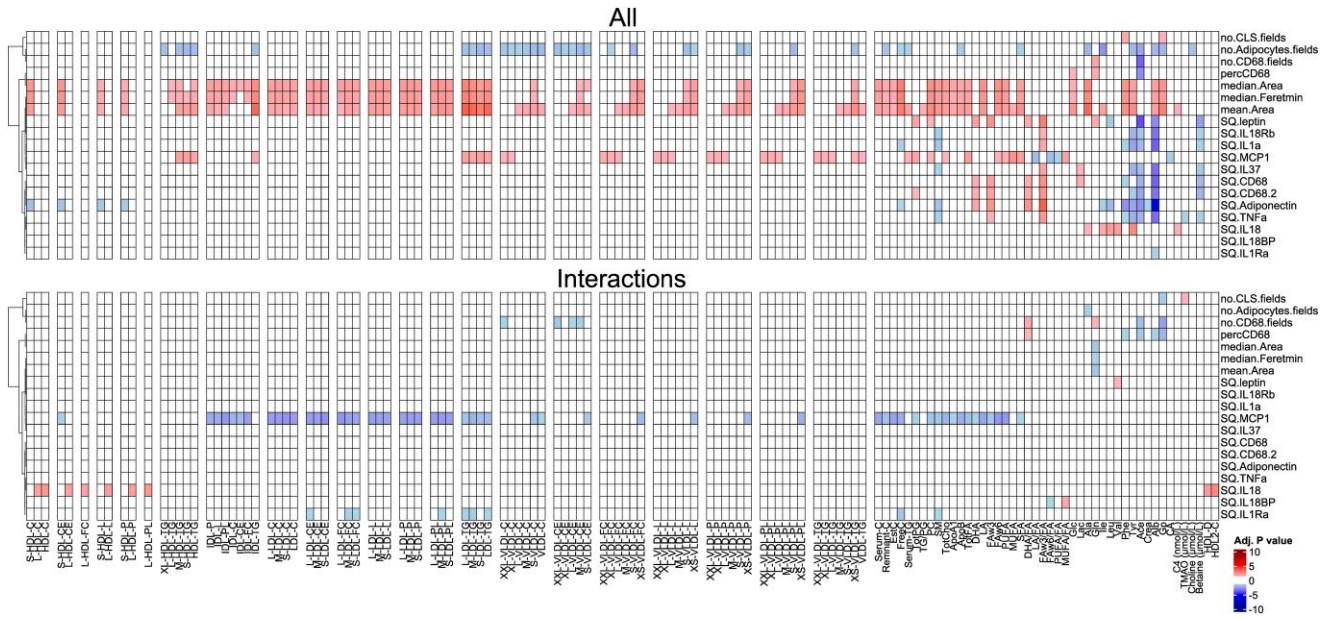

Figure 5. Association between AT inflammation and lipoprotein measures. Significant correlations (Spearman) observed between the adipose tissue parameters and lipoprotein measures assessed using the Nightingale targeted lipidomics platform; the heatmaps represent the –log10 (*P* values) multiplied by the sign of the correlation coefficient. Significance was determined using R's cor.test functionality and was adjusted for multiple testing using false discovery rate. (A) All patients combined. (B) Interaction effects measured by Fisher *Z* transformation to determine if correlations were significantly different between sexes. The color scale shows the sign of the *z* transformation multiplied against the *P* value; for positive correlations, red indicates a stronger association with women and blue a stronger in men. This is inverted with negative associations.

There were no significant associations between any of the AT parameters and circulating leukocyte numbers after multiple testing correction. Examination of scatterplots, however, shows clear trends for increased leukocytes (r_{sf} (114) = .22, P = .015; r_{sm} (139) = .17, P = .047; z = 0.46, P = .64), monocytes (r_{sf} (111) = .21, P = .025; r_{sm} (138) = .17, P = .041; z = 0.31, P = .76), eosinophils (r_{sf} (111) = .26, P = .006; r_{sm} (138) = .15, P = .075; z = 0.88, P = .38), and basophils (r_{sf} (111) = .23, P = .014; r_{sm} (138) = .17, P = .045; z = 0.5, P = .62) with increasing adipocyte size.

There were also no significant associations, in the entire cohort, between any of the AT parameters and cytokine production of PBMC after ex vivo stimulation, with the exception of several negative associations between TNF production following exposure to heat-killed *Candida albicans* and the mRNA expression of IL-1a (R_s (285) = −0.23, P = 0.048), IL-37 (R_s (285) = −0.21, P = 0.048), CD68 (R_s (285) = −0.21, P = 0.048), adiponectin (R_s (285) = −0.22, P = 0.048), and TNF (R_s (285) = −0.23, P = 0.048).

Association Between AT Inflammation and Circulating Inflammatory Markers

In addition to leukocyte numbers and function, we also assessed the interrelatedness between AT inflammation and circulating markers of inflammation.

These is a strong association in the entire cohort between adipocyte size and circulating leptin (r_s (262) = .4, P < .001; r_{sf} (118) = .24, P = .11; r_{sm} (142) = .5, P < .001; z = −2.4, P = .02), resistin (r_s (262) = .24, P = 001; r_{sf} (118) = .23, P = .11; r_{sm} (142) = .25, P = .04; z = −0.14, P = .89), and IL-18 (r_s (262) = .29, P < .001; r_{sf} (118) = .39, P = .0013; r_{sm} (142) = .23, P = .04; z = 1.35, P = .18), which also remains significant after correcting for BMI. Importantly, these associations showed strong sex specificity: for leptin, the correlation was present in men only, and circulating leptin was also associated with several markers of AT inflammation in men, including MCP-1 expression (r_{sf} (130) = −.01, P = .96; r_{sm} (149) = .25, P = .03; z = −2.21, P = .03). This sex-specific effect also resulted in a significant modulating effect of sex on the association between leptin and AT macrophage infiltration (expressed as number of CD68 cells per field; Fig. 6D). In addition, the correlation between adipocyte size and resistin concentrations was only present in men, whereas the correlation with circulating IL-18 was more pronounced in women. Interestingly, in women only, the circulating IL-18 concentration was associated with the number of CLS (Fig 6B).

Another striking sex-specific finding was the association between the amount of CLS in the AT and circulating markers of systemic inflammation, with a clear association with high specificity C-reactive protein (r_{sf} (126) = −.12, P = .42; r_{sm} (152) = .23, P = .039; z = −2.97, P = .029) and IL-6 (r_{sf} (126) = .02, P = .92; r_{sm} (152) = .22, P = .046; z = −1.7, P = .088) in men, but not in women and for IL-18 (r_{sf} (126) = .28, P = 0.048; r_{sm} (152) = .05, P = .67; z = 1.97, P = .049) in women but not in men (Fig 6B and C).

As is clearly visible in Fig. 6, much of the associations observed in the whole cohort are driven by strong associations in men. AT mRNA expression levels of various inflammatory proteins were associated with circulating protein levels of vascular endothelial growth factor, Cathepsin D activity, IL-1β, leptin, and inversely associated with concentrations of the anti-inflammatory proteins AAT and IL-18, but none of these associations was significant in women (Fig 6A-C).

We further expanded the exploration of relation between AT inflammation and circulating (inflammatory) proteins, by calculating associations with the 176 unique proteins

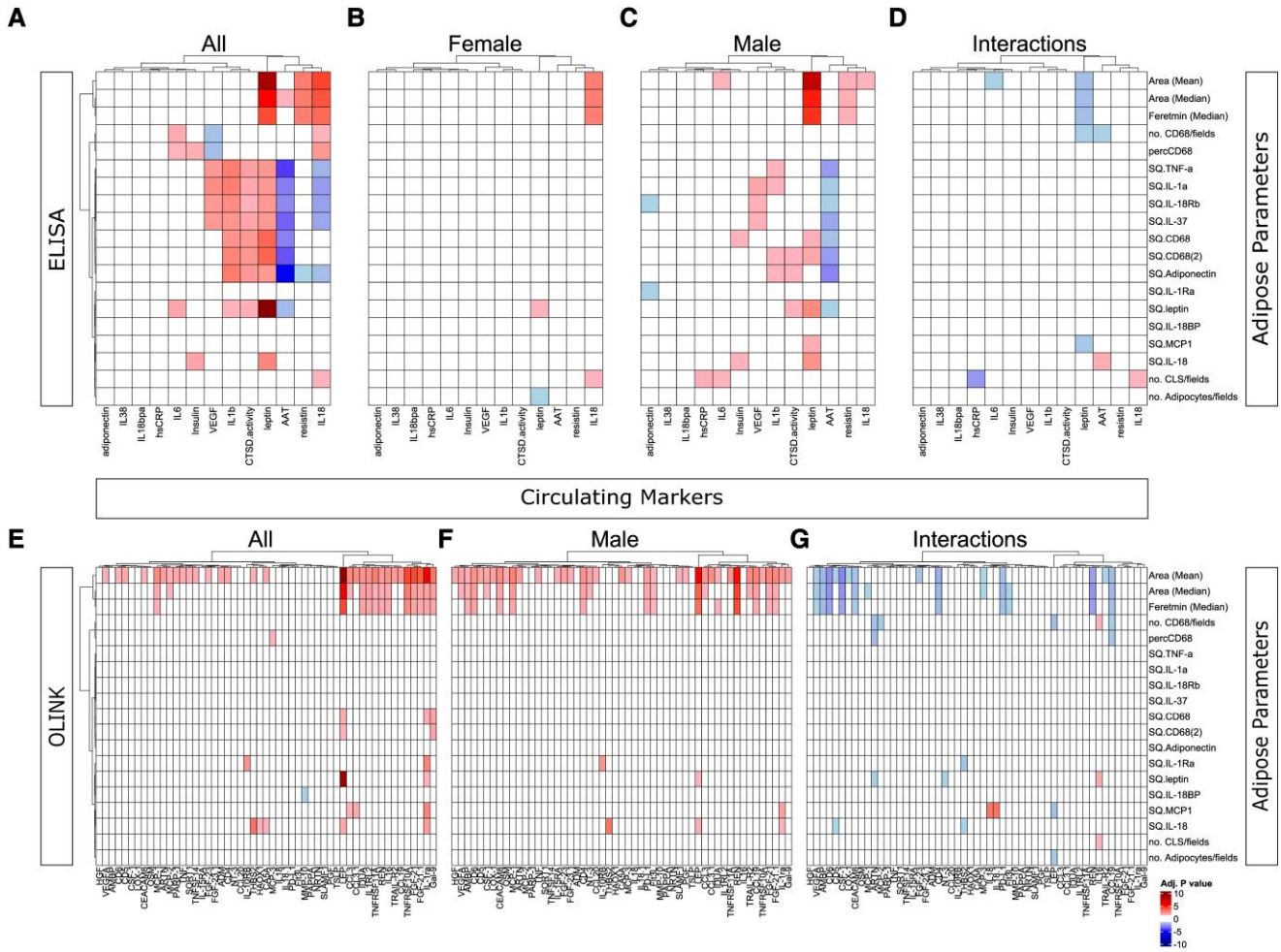

Figure 6. Association between AT inflammation and circulating inflammatory markers. Significant correlations (Spearman) observed between the adipose tissue parameters and circulating markers as measured by ELISA (A-D) and Olink (E-G). The heatmaps represent the –log10 (*P* values) multiplied by the sign of the correlation coefficient. Significance was determined using R's cor.test functionality and was adjusted for multiple testing using false discovery rate. (A, E) All patients combined. (B) Female patients only. (C, F) Male patients only. (D, G) Interaction effects measured by Fisher *Z* transformation to determine if correlations were significantly different between sexes. The color scale shows the sign of the *z* transformation multiplied against the *P* value. For positive correlations, red indicates a stronger association with women and blue a stronger in men. This is inverted with negative associations.

included in the Olink platforms Inflammation and Cardiovascular II (Fig 6E-G).

In the complete cohort, approximately 30 proteins showed an association with parameters of adipocyte size after multiple testing correction, and this was again present in men only and not in women (Fig 6E and 6F). After correction for BMI, a significant correlation remained for 10 proteins, shown in Table 3.

Because it is known that AT macrophage infiltration is a key process that drives the cardiometabolic complications of obesity, it would be beneficial to identify circulation proteins that can act as biomarkers for inflammation at the level of adipose tissue. In our cohort, after strict multiple-testing correction, only 1 protein showed a relevant association: circulating MCP-3 is correlated with the percentage of CD68 cells (R_s (278) = .2, P = .036; R_{sf} (124) = .11, P = 1; R_{sm} (152) = .26, P = .056; z = −1.24, P = .22). The MCP-1 expression in the AT, which is associated with the number of AT macrophages and CLSs, could be related to circulating concentrations of the atherogenic chemokine CCL3 (R_s (277) = .2, P = .034; R_{sf} (126) = .19, P = .88; R_{sm} (147) = .21, P = .17; z = −0.2, P = .84) and endogenous anti-inflammatory break IL-1Ra (R_s (277) = .26, P = .0021; R_{sf} (126) = .19, P = .88; R_{sm} (147) = .31, P = .012; z = −1.12, P = .26) (Fig 6E).

Discussion

Elucidation of how AT inflammation predisposes to metabolic and vascular complications in obesity could allow the identification of new pharmacological targets to prevent these complications. Previous studies are often hampered by small sample size, investigation of only a few parameters, and no systematic sex-specific analysis. We now performed these analyses in a sex-specific manner, in a large cohort of deeply phenotyped subjects with a BMI >27 kg/m^2. We previously reported important sex-specific differences in this cohort. First, we showed that men had lower volumes of superficial and deep adipose tissue (measured with MRI) than women, and only in men, deep SAT was associated with hepatic steatosis (10). In addition, we reported that men with MetS had higher circulating leptin and IL-6 concentrations, whereas women with MetS had lower anti-inflammatory adiponectin (9).

Table 3. Proteins that showed a significant association with adipocyte size (mean area) after correcting for effects of BMI

	All patients		Female patients		Male patients		Fisher *Z* score	
	R_s (260)	*P*	R_{sf} (116)	*P*	R_{sm} (142)	*P*	Z	*P*
FGF-21	0.30	<.001	0.22	.822	0.33	.018	−0.63	.527
Galectin-9	0.24	.015	0.16	.933	0.30	.056	1.14	.254
IDUA	0.22	.041	0.22	.822	0.26	.090	1.37	.171
IL-16	0.23	.041	0.20	.868	0.29	.063	0.80	.424
IL-1Ra	0.30	<.001	0.25	.570	0.27	.078	−0.21	.831
Leptin	0.38	<.001	0.30	.336	0.35	.011	1.18	.239
MCP-1	0.24	.022	0.17	.920	0.31	.042	1.08	.280
TNFRSF10A	0.27	.004	0.24	.642	0.29	.058	−1.44	.150
TNFRSF11A	0.25	.010	0.17	.927	0.33	.018	0.06	.951

The 10 proteins from the Olink panels that remained significant following correction for the effects of BMI. Spearman correlation coefficients (degrees of freedom) and *P* values are shown for the entire cohort, as well as when separating in female and male patients. Correlation coefficients between the sexes were explored using Fisher *Z* transformation and the *Z* score and *P* value are also presented here.
Abbreviation: BMI, body mass index.

In the current study, we investigated adipose tissue biopsies and focused on 3 aspects of adipose tissue inflammation: (1) adipocyte size; (2) macrophage infiltration and CLS formation; and (3) AT mRNA expression levels of several pro- and anti-inflammatory cytokines, chemokines, and adipokines. Our major findings are that there are profound sex-specific differences in the association between adipocyte size, AT inflammation, and systemic inflammation, which are much stronger in men than in women. Furthermore, AT inflammation shows a stronger association with the metabolic then with the atherosclerotic complications of obesity.

We confirmed strong associations of inflammatory parameters with adipocyte size (4) and subsequently revealed profound sex-specific differences. Adipocyte size is associated with the mRNA expression of MCP-1 and leptin and with the number of AT macrophages, only in men (Fig. 2E). In men, the association between MCP-1 expression and CLS number is stronger than in women.

In conclusion, adipocyte size appears to be a more important driver of AT inflammation and macrophage recruitment in men than in women. Importantly, a higher BMI and a higher SAT volume is associated with adipocyte size only in men, suggesting that expansion of AT occurs mainly by hypertrophy in men vs hyperplasia in women. The role of AT inflammation in the development of insulin resistance, however, was sex-independent. With regard to the development of insulin resistance, we observed associations between the number of AT macrophages and HOMA-IR and HbA1c, without clear sex differences. This confirms previous observations of associations of adipocyte size with insulin resistance (5) and the development of type 2 diabetes (20).

In addition to the metabolic complications of obesity, we explored the correlation of AT inflammation with measures of atherosclerosis, including carotid atherosclerotic plaques, carotid IMT, and pulse wave velocity. In a previous study of 109 obese individuals, the presence of CLS in the subcutaneous AT was associated with a lower flow-mediated dilation, which is a measure of endothelial function, but this study did not investigate atherosclerosis presence (7). In our study, there was an inverse association between AT mRNA expression of the anti-inflammatory mediators adiponectin and IL-37 with IMT, which is a measure for preclinical atherosclerosis. Importantly, this association was driven by the associations in men and not in women. Future research is needed to elucidate causality and underlying mechanisms. We did not observe significant associations between AT parameters and established carotid plaques, nor with pulse wave velocity. This suggests that AT inflammation is more important in the metabolic complications of obesity than in the development of atherosclerotic cardiovascular disease.

We also explored a potential role for AT IL-37 expression in AT inflammation and cardiometabolic complications of obesity. The IL-1 family member IL-37 is known to act as a natural suppressor of innate inflammation and is expressed in many cell types, including immune cells and adipocytes (21, 22). In mice with overexpression of IL-37, diet-induced AT inflammation was ameliorated, with lower MCP-1 expression and less CLS, and this was associated with less insulin resistance (22). In addition, the daily administration of recombinant human IL-37 in old mice improved endothelial vasodilator function (23). We confirmed the inverse association between IL-37 expression and MCP-1 expression and the number of CLS and observed an inverse relation between adipocyte size and IL-37 expression. Experimental studies suggest that IL-37 protects against atherosclerosis development: systemic administration of IL-37 ameliorates atherosclerosis in *ApoE*-deficient mice (24). In men, but not in women, we observed a significant inverse relation between AT IL-37 expression and carotid IMT, but not with established carotid plaques.

A particular strength of our study is that, in addition to AT parameters and clinical measures of metabolic and atherosclerotic complications of obesity, we also measured a wide spectrum of circulating inflammatory and immune parameters that could potentially mediate the effects of AT inflammation on metabolism and vasculature. It was recently hypothesized that local subcutaneous AT inflammation leads to proinflammatory cytokines and adipokines in the circulation that subsequently affect peripheral insulin sensitivity (25). This concept has only been explored in a few small studies. In 39 nondiabetic adults, AT macrophage numbers were associated with circulating TNF and C-reactive protein, which were negatively associated with insulin sensitivity (26). Experimental studies also suggest a role for AT inflammation in the activation of circulating immune cells that can subsequently promote

cardiovascular disease. In obese mice, AT macrophages produce IL-1β, which stimulates bone marrow myeloid progenitors to increase production of monocytes and neutrophils (27). Although we observed a significant association between circulating IL-1β and AT mRNA expression of various inflammatory proteins, we did not find any association with AT macrophage number or CLS number, nor did we observe any association between parameters of AT inflammation and circulating leukocyte numbers or function. Specifically, there was no association between AT parameters and circulating monocytes or neutrophils; as a result, we could not validate the mechanism proposed by Nagereddy et al in humans (27).

We observed a striking sex difference in the association between AT parameters and circulating inflammatory proteins, with most of the associations being restricted to men (Figs. 5 & 6). The most widely used markers of systemic inflammation high sensitivity C-reactive protein and IL-6 showed strong correlations with CLS number in men, but not in women. In addition, adipocyte size was strongly correlated with circulating leptin and resistin only in men, whereas in women adipocyte size and CLS number was associated with circulating IL-18. Importantly, these associations remain after correction for BMI. In addition, the circulating leptin concentration was associated with AT MCP-1 mRNA expression only in men. Finally, in the analysis of the 176 circulating proteins included in the proteomics platform, there were only positive correlations with adipocyte size in men and not women. When we specifically focused on AT parameters of macrophage infiltration, circulating MCP-3 was correlated to AT macrophage content, and CCL-3 and IL-1ra with AT MCP-1 expression. The chemokine MCP-3 is a broadly active chemoattractant that orchestrates monocytes mobilization to inflammatory sites, and its expression is upregulated in AT in obese mice (28, 29). More research is needed to explore the origin of the circulating MCP-3 in our study. In conclusion, these data argue for a stronger link between adipocyte size and AT and systemic inflammation in men than in women. This could potentially contribute to the male-specific association of adipocyte size with MetS and with our previous observation that only in men circulating leptin and IL-6 is higher in the presence of MetS (9).

Obesity and MetS are associated with dyslipoproteinemia, with mainly increased triglyceride-rich remnant lipoproteins. We explored whether circulating lipoproteins were associated with AT parameters. Interestingly, only in men, the total and LDL-C concentration was significantly correlated to AT MCP-1 expression. To explore in more detail how AT inflammation relates to circulating lipoproteins, we used the lipoprotein parameters that were measured in the Nightingale lipidomics platform. Many of the species and components of VLDL and LDL were associated with adipocyte size, and these associations remained significant after BMI correction. However, despite similar correlation coefficients in the sex-stratified analyses, the reduction in power because of fewer samples resulted in few associations being observed in each of the sexes separately. The 1 measure that showed considerable differences between sexes was MCP-1 expression in the AT, which was associated with multiple VLDL, LDL, and intermediate-density lipoprotein species, as well as total levels of cholesterols and a number of free fatty acids.

Focusing on AT macrophage infiltration, we did not observe significant associations between any of the measures of macrophage infiltration with lipoprotein-related parameters; however, there were associations observed between the percentage of macrophages with glucose and glutamine, in addition to CLS with phenylalanine and glycoprotein acetyls. The association of glycoprotein acetyls is of particular interest because this has been demonstrated to be a biomarker of chronic inflammation associated in the development of cardiovascular and cardiometabolic disease (30). This association was driven by the male patients, with a strong positive correlation in men, but a near zero correlation in women.

Our study has several limitations. First, this is an observational study, and because of the depth of the phenotyping within the cohort, independent validation poses a challenge because no other cohort provides the same wealth of data. Of particular note is that the majority of studies involving adipose tissue tend to be limited in what parameters are measured and are relatively small. Second, as a cross-sectional study, we highlight interesting avenues for further research, and further (experimental) studies are warranted to unravel causality and underlying mechanisms underpinning these associations. Third, our study was restricted to SAT, which is more abundant in women, whereas VAT is a larger fat depot in men. VAT has been described as more inflammatory than SAT and therefore may be contributing even more to the systemic inflammation than we have observed. Finally, in terms of exploring the role of AT inflammation in cardiovascular disease, it is important to realize that this cohort represents mostly "healthy" obese patients who only have asymptomatic carotid atherosclerosis. Therefore, we cannot draw any conclusions on any potential association between AT inflammation and the occurrence of cardiovascular events resulting from destabilization and rupture/erosion of plaques.

In conclusion, we show that in obese individuals, adipocyte size and AT presence of macrophages is associated with MetS and insulin resistance, but not with carotid plaques, both for men and women. Adipose tissue IL37 expression, however, was inversely associated with carotid IMT as a marker of preclinical atherosclerosis. We revealed profound sex-specific associations that urge future studies to perform sex-specific analyses: only in men, BMI is strongly associated with adipocyte size, which is correlated with AT macrophage numbers and CLS formation. In addition, only men demonstrate a strong correlation between biomarkers of AT inflammation and systemic inflammation.

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Data Availability

Original data generated and analyzed during this study are included in this published article and anonymized data and materials have been made publicly available at the Human Functional Genomics Project (HFGP) website and can be accessed at https://hfgp.bbmri.nl/.

Some datasets analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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