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ARTICLE

Clinical Research

Sex-specific association of visceral and subcutaneous adipose tissue volumes with systemic inflammation and innate immune cells in people living with obesity

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BACKGROUND AND AIMS: Obesity predisposes to metabolic and cardiovascular diseases. Adipose tissue inflammation and systemic inflammation contribute to these complications. There are strong sex differences in adipose tissue distribution and in systemic inflammation. Women have more subcutaneous adipose tissue (SAT) and less visceral adipose tissue (VAT) than men. We explored the sex differences in the association between the different adipose compartments and inflammatory markers that are important in cardiometabolic disease pathophysiology.

METHODS: Single-center observational cohort study with 302 individuals with a BMI ≥ 27 kg/m^2. We were unable to acquire MRI data from seven individuals and from another 18 the MRI data were not usable, resulting in 277 people (155 men, 122 women), aged 55–81 years.

INTERVENTION: We performed the following measurements: abdominal magnetic resonance imaging to measure VAT, and SAT (deep and superficial) volumes; circulating leukocyte counts and cytokine production capacity of peripheral blood mononuclear cells (PBMCs), circulating cytokines, adipokines, and targeted proteomics; abdominal sSAT biopsies for histology and gene expression.

RESULTS: Only in women, (s)SAT volume was associated with circulating leukocytes, monocytes, and neutrophils. Circulating IL-6 and IL-18BP were associated with SAT volume in women and VAT in men. Several circulating proteins, including monocyte-colony-stimulating factor 1 and hepatocyte growth factor, are associated with sSAT in women and VAT in men. Only in women, SAT volume is associated with SAT expression of inflammatory proteins, including leptin, CD68, TNFα and IL-1α.

CONCLUSION: In women living with obesity, abdominal SAT volume, especially sSAT, is associated with circulating leukocytes and inflammatory proteins. In men, these parameters mainly show associations with VAT volume. This could be because only in women, sSAT volume is associated with sSAT expression of inflammatory proteins. These findings underscore that future research on adipose tissue in relation to cardiometabolic and cardiovascular disease should take sex differences into account.

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INTRODUCTION

Fat distribution plays an important role in the propensity of people living with obesity to develop metabolic dysregulation (metabolic syndrome) and atherosclerotic cardiovascular disease (CVD). Visceral adipose tissue (VAT) volume is related to an increased cardiovascular risk, and non-alcoholic fatty liver disease [1]. Subcutaneous adipose tissue (SAT) can be divided into deep and superficial SAT, with dSAT reportedly having a phenotype between VAT and sSAT [2].

Fat distribution is highly sex-specific, with women having less VAT and more SAT, mainly more sSAT [3]. We recently reported differential associations for the different adipose tissue volumes with markers of metabolic dysregulation, with dSAT volume being associated with hepatic steatosis and low HDL-cholesterol in men, whereas in women with obesity, sSAT was inversely associated with hepatic fat content [3]. In addition, VAT volumes were associated with Intima-Media Thickness (IMT) only in men, but not in women [4].

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Adipose tissue inflammation, systemic inflammation, and immune cell activation is an important link between obesity and its metabolic and cardiovascular complications [5, 6].

We have previously shown profound sex-specific patterns in obesity-associated inflammation in relation to the presence of metabolic syndrome [7]. Women with metabolic syndrome had lower circulating concentrations of the anti-inflammatory adiponectin, whereas men with metabolic syndrome had higher levels of pro-inflammatory IL-6 and leptin [7]. This suggests a sex-specific role for adipose tissue on the obesity-associated low-grade inflammatory state. Until now most studies investigating the effects of fat distribution on inflammation did not perform stratified analysis investigating women and men separately [8, 9]. Many studies have shown that VAT volumes are strongly associated with cardiometabolic and cardiovascular complications in obesity, and also with circulating inflammatory markers [8, 10, 11]. For SAT, these associations are weaker, and some studies show stronger associations in women between SAT volume and circulating inflammatory markers than in men [12].

Therefore the aim of the current study is to investigate sex-specific differences in the association between the various fat compartment volumes and measures of systemic inflammation and immune cell activation in people living with overweight or obesity. Based on our previous studies, we hypothesized that VAT volumes and dSAT volumes are associated with systemic inflammation, and that these associations are stronger in men than in women.

MATERIALS AND METHODS

Study design

In this single-center observational cohort study, called the 300-Obesity (300-OB) cohort, we enrolled 302 individuals aged 55–80 with a BMI above 27 kg/m^2 at Radboud university medical center as previously described [7]. In total, 1022 individuals were contacted and 720 individuals were excluded: $n = 262$ refused to participate, $n = 458$ were excluded because they did not fulfill the inclusion criteria or had exclusion criteria. After an overnight fasting period venous blood was drawn from participants and subcutaneous adipose tissue needle biopsies were taken under local anesthesia from the abdominal compartment. To assess the abdominal fat distribution a MRI scan was conducted on a different day and evaluated by radiologists to determine the different adipose tissue volumes. In this study we could only use the data of 277 participants, because from seven individuals we could not acquire the MRI data and from another 18 individuals the MRI data was not usable (see "Results" section).

Study population

Subjects with a recent cardiovascular event (<6 months), a history of bariatric surgery or bowel resection, inflammatory bowel disease, renal dysfunction, increased bleeding tendency, use of anti-coagulant therapy, or thrombocyte aggregation inhibitors other than acetylsalicylic acid or carbasalate calcium were excluded. All lipid-lowering medication was discontinued for 4 weeks before the measurements. All participants provided written informed consent. The study was approved by the Ethical Committee of the Radboud University (nr. 34462.091.10). Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

MRI data acquisition

As previously described [13], abdominal fat distribution was determined by magnetic resonance imaging (MRI). A 3.0 T Magnetom Skyra or Trio MR system (Siemens, Erlangen, Germany) was used to perform the MR examination, with individuals in the supine position with their arms parallel to the body. Breathing commands were used to avoid motion-induced artifacts.

VAT, SAT, sSAT and dSAT analysis. The MRI images were analyzed with software developed in the IDL 6.0 environment, called HIPPO FAT (version 1.3, V. Positano) [14]. Due to the T1-weighting, fatty tissues are represented with signal intensity in these images.

VAT, SAT, dSAT and sSAT volumes were measured on eight separate slices around the L4–L5 intervertebral level, with an interslice distance of 5 mm. HIPPO FAT automatically generates three contour lines at each image provided by an active fuzzy clustering algorithm [15] that allowed the separation of SAT from VAT: (1) along the outer margin of the SAT, (2) along the inner margin of the SAT and (3) around the smallest possible region in the visceral region that included all VAT. A histogram of signal intensities in the VAT region was provided, in which a Gaussian curve automatically fitted the high-intensity peak, which identified the visceral fat.

After automatic segmentation, the analyst (TB), if necessary, manually adjusted both the contour lines and the shape of Gaussian curve by eyeballing. The MRI scan allows visualization of the scarpa fascia as a fine black line. A line was drawn manually over the scarpa fascia to divide sSAT from dSAT. Adipose tissue pixels between this line and the outer margin of the SAT were defined as sSAT. dSAT was calculated by the total subcutaneous adipose tissue pixels minus the superficial subcutaneous adipose tissue pixels (dSAT = SAT − sSAT).

Biochemical analyses

Biochemical characteristics were determined as described previously [7]. Blood sampling was performed in the morning after an overnight fast. Blood glucose, total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were measured using standard laboratory procedures, and LDL-C was calculated according to the Friedewald formula. Immune cell counts were determined in fresh whole blood EDTA samples using the Sysmex XE-5000. Plasma concentration of circulating cytokines and proteins, including high sensitivity C-reactive protein, interleukin (IL)-6, IL-18-BP, vascular endothelial growth factor (VEGF), alpha-1 antitrypsin (AAT), resistin, leptin, adiponectin (R&D/Biotechne) and IL-18 (MAGPIX), were determined using commercially available enzyme-linked immunosorbent assays (ELISA) kits.

Stimulation experiments

Whole blood (100 µl of heparin blood) was stimulated (400 µl stimulus) for 48 h at 37 °C and 5% CO_2 in a 48-well plate (Corning). Isolation of peripheral blood mononuclear cells (PBMCs) was performed as described in Oosting et al. [16].

Cytokine concentrations after stimulation were measured using commercially available ELISA kits.

Stimuli and cytokines

Data Supplementary Table 2 lists the concentrations of the stimuli used. Stimulation of PBMCs was performed with a comprehensive set of stimuli containing both purified innate immune stimuli that are associated with chronic inflammation (e.g., LPS, oxidized low-density lipoprotein (LDL)) and microorganism that are the source of microbial ligands that translocate in the circulation at the intestinal level. Interleukin (IL)-1β, IL-6, TNF-α, and IL-1Ra were measured after 24-h stimulation with these stimuli. After 7 days of stimulation with C. albicans and S. aureus: IFN-γ, IL-17, and IL-22 were measured.

Protein measurements

Circulating plasma inflammatory markers were measured with targeted proteomics as previously described [17]. In short, we used the commercially available Olink Proteomics AB (Uppsala, Sweden) Inflammation and Cardiovascular II Panel (170 different proteins), using a Proceek© Multiplex proximity extension assay [18]. In this assay proteins are recognized by pairs of antibodies coupled to cDNA strands, which bind when they are in close proximity and extend by a polymerase reaction. A pooled plasma sample and an interplate control were included on each plate in triplicate to correct for batch differences. Detected proteins are normalized and measured on a log2-scale as normalized protein expression values.

Adipose tissue analysis

After an overnight fast, subcutaneous adipose tissue biopsies were obtained under local anesthesia by needle biopsies performed 6–10 cm lateral to the umbilicus in the right lower quadrant.

Adipocyte size. The morphometry of individual fat cells was assessed using digital image analyses as described previously [19]. The adipocyte

cell diameters of all adipocytes in four microscopic fields of view were counted and measured. On average, 219 adipocytes (range 113–330) were measured per field.

Immunohistochemistry. To detect macrophages, adipose tissue sections were incubated with a CD68-monoclonal antibody (Serotec, Oxford, UK). Sections were preincubated with 20% normal horse serum followed by overnight incubation at 4 °C with the primary antibody diluted 1:40 in phosphate-buffered saline, 1% bovine serum albumin. After incubation with the primary antibody (mouse anti-human), a horse anti-mouse IgG conjugated to horseradish peroxidise (Vector labs Brunschwig) was used as a secondary antibody. Visualization of the complex was done using 3,3'-diaminobenzidene for 12 min. Negative controls were used by omitting the primary antibody. Hematoxylin and eosin staining of sections was done using standard protocols. 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 crown-like structure was defined as an adipocyte surrounded by at least three macrophages [20].

RNA isolation and qPCR analysis. Total RNA was isolated from adipose tissue using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Concentrations and purity of RNA samples were determined on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Landsmeer, The Netherlands). In total 1 µg of RNA was used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was done to study the mRNA expression levels of leptin, adiponectin, CD68, MCP-1, IL-1α, IL-1Ra, IL-18, IL-18BP, IL-18Rα, IL-18Rβ and IL-37. The gene expression of IL-1Ra, IL-18, IL-18BP and IL-18Ra were present in low quantities in the majority of the individuals, as completion of the standard curve was not possible, we did not include these genes in our analyses. The sequence-specific primer pairs of the other genes are listed in Data Supplementary Table 3. qPCR was performed by platinum Taq polymerase (Invitrogen) and SYBR Green using an iCycler PCR machine (Bio-Rad). Melt curve analysis was included to assure a single PCR product was formed. The mRNA expression of all genes reported was normalized to Ribosomal Protein L37a (RPL37A) gene expression.

Statistics

For pairwise comparisons between men and women the independent sample *t* test was used, with p value < 0.05 considered to be significant.

For the correlation analysis, we stratified the analyses for sex. Correlations and corresponding p values were calculated using the rank-based Spearman correlation as implemented in the "cor.test()" function of the "R" programming language (R Foundation for Statistical Computing, Vienna, Austria).

Regression analysis was performed using the "lm" function, which is part of the "stats" package, as implemented in the "R" language. Before the regression analysis, the circulating protein concentrations were normalized using logarithmic transformation to get a distribution more closely resembling a normal distribution. Multiple testing correction was performed using the Benjamini–Hochberg False Discovery Rate method as implemented in the "p.adjust" function of the "stats" package in the R programming language [21]. Standardized regression Coefficients (beta) were calculated based on objects of class "lm" using the "lm.beta" function part of the "QuantPsyc" package in the R programming language. The covariates included in all regression models are: age, seasonality and pack years of smoking.

RESULTS

Baseline characteristics

The 300-OB cohort consists of 302 individuals of Western-European ancestry with a BMI higher than 27 kg/m^2. We were unable to acquire MRI data in seven individuals because of

Table 1. Baseline characteristics of 300-obesity cohort.

	Total ($n = 277$)	Women ($n = 122$)	Men ($n = 155$)	p value
Sex		44%	56%	
Age (years)	67.1 ± 5.3	67.7 ± 5.3	66.6 ± 5.3	Ns
BMI (kg/m^2)	30.4 ± 3.0	30.5 ± 3.0	30.3 ± 2.9	Ns
WC (cm)	105.8 ± 9.0	101.7 ± 8.2	108.9 ± 8.4	$p < 0.001$
Pack years	21.4 ± 18.1	($n = 71$) 20.4 ± 17.3	($n = 118$) 22.1 ± 18.6	$p = 0.50$
Total Cholesterol (mmol/l)	6.3 ± 1.1	6.7 ± 1.0	6.0 ± 1.1	$p < 0.001$
LDL-C (mmol/l)	4.1 ± 1.0	4.4 ± 0.9	3.9 ± 1.0	$p < 0.001$
HDL-C (mmol/l)	1.3 ± 0.3	1.5 ± 0.30	1.2 ± 0.3	$p < 0.001$
TG (mmol/l)	1.8 ± 1.0	1.8 ± 0.8	1.9 ± 1.2	Ns
SBP (mmHg)	129 ± 14	130 ± 14	129 ± 13	Ns
DBP (mmHg)	80 ± 9	78 ± 8	82 ± 9	$p < 0.001$
Glucose (mmol/l)	5.7 ± 1.3	5.6 ± 1.2	5.7 ± 1.3	Ns
Diabetes mellitus (%)	11%	7%	13%	Ns
Hypertension (%)	59%	53%	63%	Ns
Lipid-lowering medication (%)	26%	21%	29%	Ns
Anti-hypertensive medication (%)	44%	42%	45%	Ns
Anti-diabetic medication (%)	7%	4%	10%	Ns
VAT volume (cm^3)	111.6 ± 38.3	97.5 ± 29.8	122.6 ± 40.5	$p < 0.001$
SAT volume (cm^3)	159.4 ± 52.3	190.7 ± 49.5	134.8 ± 40.0	$p < 0.001$
sSAT volume (cm^3)	69.4 ± 31.5	95.2 ± 28.4	49.1 ± 14.4	$p < 0.001$
dSAT volume (cm^3)	90.0 ± 29.7	95.5 ± 29.5	85.7 ± 29.2	$p < 0.006$
Hip SAT volume (cm^3)	116.4 ± 48.4	154.0 ± 41.8	86.8 ± 28.7	$p < 0.001$

Baseline characteristics presented as mean ± standard deviation or *n* (%) in total cohort, women and men. p value for independent sample *t* test between men and women.

WC waist circumference, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *TG* triglycerides, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *VAT* visceral adipose tissue, *SAT* subcutaneous adipose tissue, *sSAT* superficial subcutaneous adipose tissue, *dSAT* deep subcutaneous adipose tissue, *HIP SAT* subcutaneous adipose tissue at hip region, *Ns* not significant.

Table 2. Sex-specific association between fat distribution and white blood cell count and components.

(A)					
	Women				
	SAT	**SAT_hip**	**sSAT**	**dSAT**	**VAT**
Leukocytes	0.21[a]	0.15	0.20	0.16	0.12
Lymphocytes	0.05	0.06	0.09	−0.03	0.04
Neutrophils	0.20[a]	0.13	0.20	0.15	0.16
Monocytes	0.31[a]	0.22	0.22	0.27[a]	0.03
Basophils	0.25	0.05	0.15	0.27[a]	−0.03
Eosinophils	0.05	0.01	0.01	0.04	−0.08
(B)					
	Men				
	SAT				
SAT_hip	**sSAT**	**dSAT**	**VAT**		
Leukocytes	0.08	0.13	0.04	0.06	0.16
Lymphocytes	0.14	0.16	0.15	0.1	0.1
Neutrophils	0.04	0.11	0	0.03	0.14
Monocytes	0.06	0.09	0	0.05	0.16
Basophils	0.03	0.03	−0.01	0.04	0.05
Eosinophils	−0.02	0.07	−0.03	−0.03	0.08

Correlation between adipose tissue compartment volumes and white blood cell count and component in women (A) and men (B). Standardized betas after adjustment for age, pack years and season.
p value: [a] $p < 0.05$.

claustrophobia. We were unable to calculate VAT due to insufficient delineation of intra-abdominal organs on the MRI images or due to movement artefacts in 18 subjects leaving 277 subjects (122 women and 155 men) for analysis of all abdominal adipose tissue compartments. As reported previously [3], in this cohort although the BMI did not differ between women and men, women had higher volume of total SAT, sSAT and dSAT and less VAT volume compared to men. Baseline characteristics are shown in Table 1.

There are significant differences in systemic inflammation between men and women, with women having higher circulating hsCRP (3.6 vs. 2.3 ug/ml, $p < 0.001$). The circulating monocyte number was significantly lower in women, although the difference was small (0.53 vs. 0.46×10^9/l, $p < 0.001$). Finally, as expected, women had higher leptin (25.4 vs. 12.0 ng/ml, $p < 0.001$) and adiponectin concentrations (6.0 vs. 3.6 ug/ml, $p > 0.001$; Data Supplementary Table 1).

Sex specific correlations between adipose tissue volumes and circulating inflammatory parameters

Association with white blood cell count. Circulating monocytes and neutrophils importantly contribute to the development of atherosclerotic CVD [22]. Therefore, we first explored the associations between circulating leukocyte numbers and the various adipose tissue volumes.

Table 2A shows that in women, total SAT volume was associated with circulating total leukocyte number, and also neutrophil and monocyte number. Monocytes and basophils were also associated with dSAT volumes. In contrast, in men there were no associations with circulating leukocyte numbers (Table 2B).

Association with circulating adipokines and cytokines. Leptin levels are strongly associated with SAT volumes in both women and men with standardized β of 0.57 vs. 0.60 respectively, $p < 0.0001$, and a similar association is shown for leptin with VAT in both women and men (Table 3A, B). In contrast, adiponectin is negatively associated with VAT in women and men (standardized $\beta = -0.38$ vs. -0.28 respectively, $p < 0.01$).

Several circulating cytokines also show sex-specific associations with fat compartments. Levels of IL-6 were associated in women with SAT, SAT hip and sSAT volumes (standardized $\beta = 0.22$, 0.27 and 0.24 respectively, $p < 0.05$), whereas in men IL-6 was also related to VAT and dSAT volumes (standardized $\beta = 0.27$ $p < 0.01$).

IL-18 and IL-18BP (binding protein) affect the development of insulin resistance in obesity [23]. In women IL-18BP levels were related to SAT (standardized $\beta = 0.25$ $p < 0.05$), while in men these levels were not related to any of the compartments. The levels of hsCRP showed an association with the SAT hip only in men (standardized $\beta = 0.23$ $p < 0.05$).

Association with cytokine production capacity. Cytokine production capacity of PBMC's is an indicator of the intrinsic inflammatory responsiveness and has previously been related to the presence of atherosclerosis [24, 25]. In our cohort we did not see associations between cytokine production capacity and adipose tissue compartment volumes (Data Supplementary Table 4).

Association with circulating proteome. We subsequently explored the association between the various fat depot volumes and the circulating protein levels of 170 different proteins using OLINK Inflammation and Cardiovascular II panels (Table 4).

Supporting our other findings, most inflammatory proteins were positively related to the amount of SAT (sSAT and or dSAT) in women, whereas in men mainly the VAT showed positive associations and there were no associations at all with the sSAT volume.

Again, leptin showed the strongest association with all SAT compartments for both sexes (standardized $\beta = 0.56$ vs. 0.57 respectively, $p < 0.000001$), and in men also with VAT (standardized $\beta = 0.46$ with $p < 0.000001$). Hepatocyte growth factor (HGF) and macrophage colony-stimulating factor1 (CSF1) in women are strongly related to SAT and sSAT, while in men with dSAT and only

Table 3. Sex-specific association between fat distribution and circulating cytokines.

(A)					
	Women				
	SAT				
SAT_hip	**sSAT**	**dSAT**	**VAT**		
hsCRP	0.20	0.18	0.16	0.18	0.17
IL-6	0.22[a]	0.27[a]	0.24[a]	0.16	0.15
IL-18	0.04	0.01	−0.02	0.08	0.00
IL-18BP	0.25[a]	0.14	0.20	0.22[a]	0.09
VEGF	0.04	−0.03	−0.02	0.09	0.12
AAT	−0.09	−0.04	0.03	−0.17	0.07
Resistin	0.07	−0.01	0.15	−0.04	0.00
Leptin	0.57[c]	0.44[c]	0.46[c]	0.50[c]	0.25[a]
Adiponectin	−0.02	−0.04	0.00	−0.02	−0.38[b]
(B)					
	Men				
	SAT				
SAT_hip	**sSAT**	**dSAT**	**VAT**		
hsCRP	0.17	0.23[a]	0.13	0.17	0.18
IL-6	0.28[b]	0.32[b]	0.22[b]	0.27[b]	0.27[b]
IL-18	0.08	0.07	0.06	0.07	0.05
IL-18BP	0.00	0.00	−0.03	0.00	0.16
VEGF	0.02	−0.04	0.00	0.02	0.01
AAT	0.10	0.05	0.04	0.12	−0.05
Resistin	0.13	0.11	0.12	0.12	−0.07
Leptin	0.60[c]	0.60[c]	0.46[c]	0.57[c]	0.43[c]
Adiponectin	−0.02	0.02	0.00	−0.02	−0.28[b]

Correlation between different adipose tissue compartment volumes and a panel of circulating inflammatory mediators in women (A) and men (B). Standardized betas after adjustment for age, pack years and season.
hsCRP high sensitivity C-reactive protein, *IL-* Interleukin-, *VEGF* vascular endothelial growth factor, *AAT* alpha-1 antitrypsin.
p value: [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.0001$.

HGF with VAT. CCL19 was significantly associated with VAT in men and SAT in women (standardized $\beta = 0.26$ and 0.29 respectively, $p < 0.05$).

In women, many proteins show strong positive associations with SAT compartments, including various proteins related to the TNF signaling pathway, such as Tumor necrosis factor receptor superfamily member 9 (TNFRSF9), 10A, 11A, and the receptor for TNF-related apoptosis-inducing ligand (TRAIL-R2). In men, various proteins only show an association with VAT.

In conclusion, sex-specific differences are present in the relation between the various fat compartments and circulating inflammatory mediators and immune cells. The most consistent finding is that SAT volume is related to monocyte and neutrophil numbers only in women. In addition, circulating inflammatory proteins were associated mainly to SAT and sSAT in women, and in men mostly to VAT volumes. Finally, we explored sex-differences in the association between adipose tissue volumes and adipose tissue biopsies.

Sex-specific adipose tissue distribution in relation to adipose tissue inflammation

The fat biopsies were taken from the abdominal sSAT compartment. Interestingly, we observed a strong association in women between SAT and sSAT volumes and the tissue expression levels of adiponectin, leptin, CD68, TNFα, IL1α, IL18Rβ, IL37 (Table 5). In men, these association were not significant. In addition, the size of adipocytes showed strong associations with SAT, Hip SAT, sSAT and dSAT in men, but showed no significant associations in women.

DISCUSSION

In the present study, we found clear sex-specific differences in the association between adipose tissue distribution and circulating markers of chronic low-grade inflammation, and circulating leukocytes in people living with overweight or obesity (BMI > 27 kg/m^2). Unexpectedly, we observed that in women SAT volume is related to white blood cell count, circulating monocytes and neutrophils, and circulating levels of many inflammatory proteins, including IL-6, IL-18bp, TRAILR2, LOX1, and CEACAM8. In contrast, in men there was no association of adipose tissue volumes with circulating leukocytes, and circulating levels of inflammatory proteins were mostly strongly associated with VAT and not SAT volumes. Detailed analysis of sSAT biopsies revealed that a higher sSAT volume is associated with higher gene expression of inflammatory proteins, and that this association is stronger in women than in men. We propose that this mechanism, together with the much higher sSAT volumes in women, contributes to the observed associations between sSAT volumes and circulating inflammatory parameters in women more than in men. These findings call for a sex-specific approach in future studies focussing on inflammation in people living with obesity.

Our results confirmed strong sex differences in systemic inflammation, with significantly higher hsCRP in women

Table 4. Associations between circulating protein levels and adipose tissue compartment volumes in women and men.

Table 4	Women					Men				
	SAT	HIP SAT	sSAT	dSAT	VAT	SAT	HIP SAT	sSAT	dSAT	VAT
CCL19	0.29	0.28	0.29	0.22	0.23	0.16	0.22	0.07	0.16	0.26
HGF	0.36	0.26	0.36	0.25	0.29	0.26	0.25	0.11	0.31	0.34
CSF_1	0.36	0.37	0.41	0.20	0.07	0.20	0.18	0.12	0.21	0.24
ADM	0.32	0.29	0.22	0.31	0.16	0.25	0.24	0.20	0.24	0.12
LEP	0.56	0.42	0.47	0.47	0.19	0.57	0.57	0.40	0.57	0.46
THBS2	0.21	0.27	0.22	0.11	0.27	0.21	0.17	0.11	0.22	0.21
IL_15RA	0.33	0.29	0.29	0.27	0.06	0.12	0.12	0.08	0.13	0.10
IL_12B	0.29	0.30	0.30	0.20	0.22	0.07	0.07	0.06	0.09	0.09
CXCL10	0.21	0.29	0.24	0.09	0.12	0.02	0.02	–0.02	0.04	0.15
TNFRSF9	0.34	0.26	0.33	0.25	0.13	0.08	0.11	0.07	0.08	0.11
PGF	0.30	0.22	0.24	0.28	0.10	0.02	–0.01	–0.01	0.05	0.15
TNFRSF10A	0.32	0.16	0.19	0.33	0.25	0.10	0.16	0.08	0.10	0.13
TNFRSF11A	0.42	0.33	0.42	0.30	0.14	0.10	0.07	0.10	0.08	0.17
TRAIL_R2	0.36	0.26	0.34	0.27	0.09	0.15	0.18	0.19	0.11	0.11
LOX_1	0.31	0.14	0.23	0.31	0.09	0.11	0.16	0.06	0.09	0.04
SPON2	0.29	0.22	0.17	0.30	0.04	0.11	0.11	0.10	0.11	0.17
GH	–0.07	–0.06	–0.10	0.01	–0.31	0.06	0.07	0.09	0.04	–0.24
TM	0.34	0.21	0.25	0.33	–0.07	–0.05	–0.08	–0.05	–0.04	–0.05
IL16	0.37	0.29	0.26	0.39	–0.02	0.00	–0.03	0.04	–0.04	–0.02
CEACAM8	0.37	0.20	0.37	0.28	–0.01	0.13	0.12	0.17	0.08	0.06
LPL	0.24	0.17	0.17	0.25	–0.25	0.21	0.10	0.23	0.18	–0.15
CTSL1	0.25	0.28	0.21	0.23	0.14	0.06	0.04	0.04	0.05	0.05
hOSCAR	0.31	0.20	0.25	0.28	0.05	–0.02	0.04	0.03	–0.03	0.07
PARP_1	0.16	0.02	0.01	0.23	0.09	0.02	0.04	–0.02	0.02	0.25
IL_1ra	0.25	0.22	0.17	0.23	0.17	0.29	0.32	0.20	0.27	0.31
FGF_23	0.24	0.21	0.14	0.25	0.15	0.24	0.17	0.19	0.26	0.09
HAOX1	0.25	0.27	0.17	0.25	0.30	–0.05	–0.08	–0.12	–0.01	0.37
IL6	0.26	0.31	0.29	0.16	0.20	0.27	0.33	0.19	0.27	0.29
FGF_21	0.15	0.07	0.09	0.14	0.12	0.08	0.09	0.01	0.11	0.24
SCF	0.00	0.02	–0.09	0.09	0.20	–0.14	–0.13	–0.07	–0.15	–0.28
REN	0.20	0.20	0.22	0.12	0.07	0.23	0.17	0.10	0.26	0.27
KIM1	0.03	0.00	–0.06	0.10	0.14	–0.01	–0.01	–0.05	0.01	0.27
GT	0.00	0.04	0.08	–0.08	–0.09	0.00	–0.03	0.06	–0.02	–0.22
ACE2	0.17	0.18	0.15	0.13	0.12	0.02	0.05	–0.08	0.06	0.31
CA5A	0.17	0.14	0.09	0.19	0.24	0.10	0.07	0.07	0.11	0.37
CD4	0.28	0.29	0.25	0.22	0.13	0.22	0.23	0.14	0.24	0.24
VEGFD	0.08	–0.08	0.04	0.11	–0.19	–0.28	–0.32	–0.24	–0.28	–0.25

P–value	
	p <0.05
	p <0.01
	p <0.001
	p <0.0001
	p <0.00001
	p <0.000001

Heatmap of correlation between circulating protein levels in relation to different adipose tissue compartment volumes in women and men. Standardized betas after adjustment for age, pack years and season and multiple testing. The intensity of the color represent the strength of the significance.
TNFRSF tumor necrosis factor receptor superfamily member, *TRAIL-R2* TNF-related apoptosis-inducing ligand receptor 2, *HGF* hepatocyte growth factor, *CSF1* macrophage colony-stimulating factor 1, *CCL19* C-C motif chemokine 19, *LOX1* lectin-like oxidized LDL receptor 1, *CEAMCAM8* carcinoembryonic antigen-related cell adhesion molecule 8.

Table 5. Relation between fat distribution and adipose tissue histology and gene expression in women and men.

(A)					
Histology	**Women**				
	SAT	**HIP SAT**	**sSAT**	**dSAT**	**VAT**
Adipocyte cell size	0.10	0.13	0.10	0.06	0.07
CD68%	−0.08	0.00	−0.02	−0.16	0.12
CLS	−0.17	−0.16	−0.13	−0.19	0.17
Gene expression					
Adiponectin	0.18	0.23[a]	0.21[a]	0.12	0.08
Leptin	0.22[a]	0.16	0.22[a]	0.17	0.04
CD68	0.18	0.28[a]	0.28[a]	0.08	0.25[a]
MCP1	−0.12	−0.14	−0.14	−0.09	0.07
TNF	0.18	0.27[a]	0.27[a]	0.08	0.18
IL1α	0.16	0.22[a]	0.23[a]	0.07	0.15
IL18Rβ	0.18	0.25[a]	0.24[a]	0.09	0.18
IL37	0.20	0.26[a]	0.28[a]	0.09	0.17
(B)					
Histology	**Men**				
	SAT	**HIP SAT**	**sSAT**	**dSAT**	**VAT**
Adipocyte cell size	0.29[b]	0.23[a]	0.19[a]	0.30[b]	0.21
CD68%	−0.06	−0.07	−0.11	−0.05	0.04
CLS	−0.11	−0.11	−0.22	−0.05	0.10
Gene expression					
Adiponectin	0.07	0.11	0.24	−0.02	−0.07
Leptin	0.14	0.23	0.19	0.10	0.16
CD68	0.06	0.11	0.21	−0.02	0.04
MCP1	0.02	0.14	−0.02	0.02	0.20
TNF	0.07	0.04	0.18	0.01	0.05
IL1α	0.09	0.09	0.22	0.02	0.03
IL18Rβ	0.09	0.08	0.20	0.03	0.06
IL37	0.09	0.07	0.21	0.03	0.05

Correlation between adipose tissue histology and adipose tissue gene expression (after normalization to Ribosomal Protein L37a (RPL37A) gene expression) and different adipose tissue compartment volumes in women (A) and men (B). Standardized betas after adjustment for age, pack years and season.
CLS crown-like structure, *MCP1* monocyte chemoattractant protein 1, *TNF* tumor necrosis factor, *IL-* interleukin-, *IL18Rβ* interleukin 18 receptor β.
p value: [a]$p < 0.05$; [b]$p < 0.005$.

compared to men [8, 26]. A number of studies investigated the contributing role of VAT and SAT volumes on markers of low-grade inflammation, and on metabolic and cardiovascular complications of obesity [27–30]. Less data have been published on the superficial and deep SAT compartments. It is known that the dSAT adipocytes are more lipolytic than the sSAT adipocytes, proving more free fatty acids into the circulation [31]. Furthermore dSAT probably exhibits an intermediate phenotype between VAT and sSAT [32, 33]. In many observational studies, VAT mass is strongly correlated with cardiometabolic and cardiovascular complications of obesity [34]. In some studies, SAT volumes are also associated with cardiometabolic dysregulation but to a lesser extent [35], or even appear to be protective. Neeland et al. reported that SAT volumes were not related to atherosclerosis, but did relate with circulating leptin and inflammatory biomarkers [1]. Some studies even suggest beneficial effects of sSAT [36]; in patients with type 2 diabetes mellitus, a higher relative distribution of abdominal fat in sSAT was associated with a lower cardiovascular risk [37, 38], whereas dSAT was related to a higher blood pressure and lower heart rate variability [39]. However, this study population mainly consisted of men and a sex-stratified analysis to investigate sex-specific differences was performed only in a minority of studies, which were population-based and not selected on high BMI, like in our cohort. In 1250 patients from the Framingham Heart risk study, VAT and SAT volumes contributed similarly to CRP in men and women, while the contribution of both compartments in women was higher compared to men [8]. In another large cohort of healthy elderly (Health ABC), it was found that after adjustment for BMI, only VAT volume was associated with higher IL-6 and CRP concentrations in both men and women [40]. A larger contribution of SAT to a pro-inflammatory status in women has previously been described. In a group of 208 healthy men (42 years, BMI 26.0) and 145 healthy women (37 years, BMI 26.6) CRP concentrations in men were largely influenced by VAT, whereas SAT was the key correlate of CRP in women [41].

In our study, we extend these findings by showing that these sex differences are not only restricted to associations with systemic inflammatory markers such as CRP, but can be found for circulating leukocyte counts and an extensive panel of

circulating inflammatory markers. Also, we describe that these associations are driven not only by dSAT but also sSAT volume in women, suggesting that in women, sSAT volume is not benign and can induce systemic inflammation, which could potentially contribute to cardiometabolic complications of obesity. This fits with the previous observation in a cohort of healthy subjects that in women, CRP had a stronger association with SAT volume than in men, although this study did nor separate SAT into superficial and deep depots [41]. Importantly, we observed significant positive associations in women between sSAT volume and adipose tissue expression of various pro-inflammatory markers (CD68, TNF-α, IL-1α, IL-18Rβ) and also the anti-inflammatory IL-37, whereas in men these associations were not significant. Along with the fact that sSAT volume is much larger in women than in men (95 cm^3 vs. 49 cm^3), this could have contributed to the observed associations in women between sSAT and systemic inflammation, but future studies are required to confirm this. Another potential mechanism that could contribute to the observed sex-differences is the regulating effect of Treg cells in adipose tissue. Within adipose tissue, Treg cells can suppress adipose tissue inflammation [42] and there are marked sex-specific differences in adipose tissue Treg cell numbers [43]. Future studies are needed to elucidate any role of Treg cells in the sex-specific associations between adipose tissue volumes and systemic inflammation.

We observed positive associations in women between SAT volumes and circulating leukocyte, monocyte, neutrophil, and basophil numbers. In men, there were no significant correlations. The relation between obesity, and visceral fat in particular, and leukocyte counts is well established [44, 45], but to our knowledge this has not been studied for subcutaneous adipose tissue specifically. Animal studies have reported that the association between SAT and leukocyte counts might be caused by adipose tissue or adipose tissue macrophage-derived factors, such as IL-1b, that induce myeloid progenitor cell proliferation and expansion [46]. Interestingly, we observed strong positive associations between circulating M-CSF (CSF_1 in Table 4) and SAT and sSAT volumes in women, whereas in men there was no significant association with SAT or sSAT, but only dSAT. M-CSF is a pro-inflammatory cytokine that regulates differentiation, proliferation, and survival of monocytic progenitor cells, and also plays a role in monocyte-to-macrophage differentiation in the atherosclerotic plaque. Similarly, the circulating hepatocyte growth factor (HGF) concentration was associated with SAT and sSAT volume in women and both VAT/SAT, but not sSAT volumes in men. HGF is known to stimulate growth of hematopoietic progenitor cells [47], and is a known risk factor for coronary heart disease, heart failure, stroke, and progression of atherosclerosis [48]. Elevated serum HGF has been found in the context of several CVD risk factors, including obesity and diabetes [49]. Both the M-CSF and HGF have been shown to be produced in the adipose tissue [48, 50].

With regard to the targeted proteomics analyses, we observed differences between the associations with adipose tissue volumes in men and women. In women, the majority of significant associations was with SAT (deep and/or superficial), whereas in men, the majority of associations was restricted to VAT volumes (Table 4). We highlight a few of these differential associations. In women LOX-1, which is a receptor for oxidized LDL, showed association with SAT volume. Adipocytes express low LOX-1, however adipose tissue resident macrophages do express LOX-1 [51, 52] and obesity is related to higher circulating soluble LOX-1 [53, 54]. Furthermore LOX-1 has been found in carotid atherosclerotic lesions [55, 56] and the level of LOX-1 in the circulation might be predictive for cardiovascular risk [57, 58]. In addition, various proteins related to the TNF signaling pathway (TNFRSF9, 10A, 11A, and TRAIL-R2) were strongly associated with SAT volumes in women, without showing any association in men. TNF levels are increased in obesity and suggested to play a role in insulin resistance [6]. TNFRSF9 and TRAIL-R2 are independent predictors of cardiovascular events in patients with stable coronary artery disease [59]. In people living with obesity, TNFRSF10A is associated with in increased risk of heart failure [60]. TNFRSF10A and TRAIL-R2 are both receptors for TRAIL, a member of the TNF protein superfamily, that can protect against the development of diabetes mellitus [61].

Limitations

Due to the cross-sectional design of our study, no conclusion can be drawn on causality and our main findings need further validation in independent cohorts. As we only include individuals of Western European ancestry, extrapolation of our results to other ethnic groups are uncertain. Furthermore, as we included individuals who were 55–80 years of age and all women were postmenopausal, our results cannot be extrapolated to other age groups.

CONCLUSION

In the present study, we showed in a cohort of subjects with a BMI ≥ 27 kg/m^2 that SAT volume in women, in contrast to men, is associated with circulating leukocyte numbers. The concentration of various inflammatory proteins show strong sex-specific associations with adipose tissue volumes, with associations with VAT in men and with SAT, sSAT and dSAT in women. Abdominal SAT volume is linked to the tissue expression of inflammatory proteins in adipose tissue, and this is significant only in women and not in men. These findings underscore that future research on adipose tissue in relation to cardiometabolic and cardiovascular disease, should always take into account sex.

DATA AVAILABILITY

The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

ICLM and HB: drafting of the manuscript, data acquisition, analysis, equal contribution. KS and TB: data acquisition. RH, MvdG, and HMD: data acquisition and analysis. RS, JG, LABJ, MGN, NPR, and JHWR: study design and funding, supervision of data acquisition and analyses. All co-authors contributed to writing of the manuscript. All authors provided critical revisions for important intellectual content, approved the final version submitted for publication, and agreed to be accountable for all aspects of the work.

COMPETING INTERESTS

LABJ and MGN declare that they are scientific founders of Trained Therapeutics Discovery (TTxD) and LEMBA therapeutics. All other authors declare no competing interests.

ADDITIONAL INFORMATION

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