Relevance of Omental Pericellular Adipose Tissue Collagen in the Pathophysiology of Human Abdominal Obesity and Related Cardiometabolic Risk

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ABSTRACT

Background: Adipose tissue fibrosis is a relatively new notion and its relationship with visceral obesity and cardiometabolic alterations remains unclear particularly in moderate obesity. **Objective:** Our objective was to examine if total and pericellular collagen accumulation are relevant for the pathophysiology of visceral obesity and related cardiometabolic risk. **Subjects and methods:** Omental (OM) and subcutaneous (SC) fat surgical samples were obtained in 56 women (age: 47.2±5.8 years; BMI: 27.1±4.4kg/m^2). Body composition and fat distribution were measured by dual-energy x-ray absorptiometry and computed tomography, respectively. Total and pericellular collagen were measured using picrosirius red staining and CD68+ cells, a marker of total macrophages, as well as CD163+ cells, a marker of M2-macrophages, were identified using immunohistochemistry. **Results:** We found that only pericellular collagen percentage, especially in OM fat, was associated with higher BMI, body fat mass and adipose tissue areas as well as lower radiologic attenuation of VAT and altered cardiometabolic risk variables. Strong correlations between peri-adipocyte collagen percentage and total or M2-macrophages percentage were observed in both depots. Total collagen percentage in either compartment was not related to adiposity, fat distribution or cardiometabolic risk. **Conclusion:** As opposed to whole-tissue based assessments of adipose tissue fibrosis, collagen deposition around the adipocyte, especially in the OM fat compartment is related to total and regional adiposity as well as altered cardiometabolic risk profile.

Keywords: Total and pericellular collagen percentage, fibrosis, abdominal adipose tissue, macrophage infiltration

INTRODUCTION

Robust clinical and epidemiological studies now support the notion that excess fat accumulation within the abdominal cavity is strongly associated with metabolic alterations such as insulin resistance, type 2 diabetes, dyslipidemia and low-grade, chronic inflammation (1-5). Adipose tissue dysfunction, leading to limited lipid storage and reduced adipose tissue expandability, represents one of the major mechanisms to explain the link between visceral obesity and metabolic alterations (3).

In response to a chronic positive energy imbalance, adipose tissue expands by increasing the size of mature adipocytes (adipose tissue hypertrophy) and/or by generating new adipocytes (adipose tissue hyperplasia) (6-8). These morphological changes lead to immune cell accumulation and extracellular matrix (ECM) remodeling in adipose tissue (9). ECM components such as collagens and adhesion proteins provide structural support and are essential to maintain adipocyte function and integrity (10-13). Under normal physiological conditions, adipose tissue growth seems to be related to dynamic ECM remodeling, while under pathological conditions such as obesity, an imbalance in collagen synthesis and degradation may result in excessive accumulation of ECM components and later fibrosis (14).

Even if the presence and pathophysiological impact of fibrosis has been extensively examined in organs such as the liver, the existence of fibrosis in human adipose tissues is a relatively new notion and its pathological impact on adipose tissue biology remains to be understood (12, 15). In animal models, Khan et al. (16) demonstrated that collagen VI-null *ob/ob* mice were characterized by larger adipocytes, substantial improvements of insulin sensitivity as well as lower inflammatory markers compared to *ob/ob* mice, suggesting that ECM may limit adipocyte expansion during obesity thereby leading to ectopic lipid accumulation. For the first time in

humans, interstitial fibrosis was described in SC adipose tissue of severely obese subjects (17). In that study, the expression of many genes encoding ECM components in adipose tissue was dysregulated in obesity (17). Picrosirius red staining, a marker of fibrillar collagens, showed various fibrosis patterns in SC and visceral depots and revealed the importance of pericellular fibrosis (i.e. collagen surrounding adipocytes) in obese compared to lean subjects in abdominal fat compartments (18).

Pasarica et al. (19) reported that collagen VI mRNA expression in SC adipose tissue was positively associated with BMI, body fat mass, visceral adipose tissue mass and macrophage markers. Other studies also showed that collagen accumulation was not always deleterious and may actually reflect the physiological adaptation of ECM in dynamic situations such as child growth (20, 21) or body weight changes (22, 23). These differences among studies may be explained by population characteristics and physiological conditions, variations in the methodology used to measure collagen accumulation (red picrosiris staining, Masson's trichrome staining and expression or staining of collagen species) and fat depot specificities (22). Most importantly, total and pericellular collagen measurements may not necessarily be equivalent. To our knowledge, no study has ever examined the link between total and pericellular collagen accumulation in human adipose tissues and detailed body fat distribution. Considering that excess ECM may limit adipose tissue expandability (16) and that visceral fat accumulation is strongly associated with metabolic alterations (3), depot-specific differences in total and/or pericellular collagen accumulation may have pathophysiological relevance in the development of obesity-related cardiometabolic alterations.

The main objective of the study was to better understand regional differences in total and pericellular collagen accumulation and to determine which of these measurements is more

relevant to abdominal obesity, inflammation and cardiometabolic alterations in women. We tested the hypothesis that peri-adipocyte collagen is associated with higher obesity level and abdominal fat accumulation, lower adipose tissue attenuation and altered cardiometabolic risk factors. We also tested the hypothesis that pericellular collagen accumulation is closely related to macrophage accumulation.

SUBJECTS AND METHODS

Participant recruitment

Participants were recruited through the elective surgery schedule of the Gynecology Unit of CHU de Québec-Laval University Medical Center. The study sample included 56 otherwise healthy women who elected for total (n=53) or subtotal (n=2) abdominal hysterectomies or myomectomy (n=1). The study was approved by the Research Ethics Committees of Laval University Medical Center (C09-08-086) and all subjects provided written informed consent before their inclusion in the study.

Anthropometrics, body composition and body fat distribution measurements

On the morning of surgery, body weight, height, body mass index (BMI) and waist circumference were measured using standardized procedures. Body fat distribution and body composition measurements were performed a few days before the surgery. Body composition was assessed by a dual energy X-ray absorptiometry (DXA) (Hologic QDR-4500A densitometer with whole-body fan beam software v8.26a:3, Hologic, Bedford, MA, USA). Abdominal subcutaneous (SAT) and visceral adipose tissue (VAT) cross-sectional areas at the L4-L5 vertebrae level were determined by computed tomography using a GE Light Speed 1.1 CT scanner (General Electric Medical Systems, Milwaukee, WI, USA), as previously described (24, 25). Considering the ability of each tissues to attenuate X-rays, this technique distinguishes fat tissue from bone and muscle tissues. Regions of interest were delineated with the ImageJ 1.33u software (National Institutes of Health, Bethesda, ND, USA). Scan images were used for the quantification of adipose tissue areas using an attenuation range of -190 to -30 Hounsfield Units. Mean SAT and VAT attenuation were determined using four abdominal CT scans for each participant. Attenuation values are expressed in Hounsfield units (HUs).

Plasma lipid profile, glucose homeostasis and inflammatory markers

After a 12h-overnight fast, fasting blood samples were collected on the morning of surgery. Cholesterol and triglyceride levels in plasma and lipoprotein fractions were measured as previously described (25). Glucose was measured with a Modular P800 system (Roche Diagnostics, Laval, Canada). Insulin was measured by ELISA (Alpco, Salem, NH, USA). HOMA insulin resistance index was calculated from fasting glucose and insulin levels (26). Plasma IL-6, leptin and adiponectin levels were measured by commercially available ELISA (Human IL-6 Quantikine HS ELISA, R&D Systems; Minneapolis, MN, USA; Human Leptin ELISA kit, EMD Millipore; Billerica, MA, USA; Human Adiponectin ELISA Kit, B-Bridge International Inc, Santa Clara, CA, USA). Plasma CRP levels were measured in plasma using the Behring Latex-Enhanced highly sensitive CRP (hs-CRP) assay on a Behring Nephelometer BN-100 (Behring Diagnostic, Westwood, MA) and the calibrators (N Rheumatology Standards SL) provided by the manufacturer.

Adipose tissue samples

SC and OM adipose tissue samples were collected during the surgical procedure at the site of incision (lower abdomen) and at the distal portion of the greater omentum, respectively. Samples were immediately carried to the laboratory. To measure adipocyte diameter, collagen and macrophage infiltration by immunohistochemistry, a portion of the sample was fixed in 10% formalin and then processed for standard paraffin embedding. Five-micrometer slices of SC and OM adipose tissues were mounted on the same slide for each patient and were treated as described below.

Adipocyte diameter and collagen measurements

Adipose tissue slides were stained with picrosirius red for collagen quantification (17, 18) and haematoxylin/eosin for adipocyte diameter measurements. Digital slides were obtained by scanning total sample area at 20X magnification and resolution 0.24 µm/pixel using a NanoZoomer Hamamatsu scanner (Hamamatsu Photonics KK, Systems Division). Mean adipocyte diameter was measured for an average of 100 adipocytes per sample, as previously described (27). Collagen analysis was performed using CaloPix software (Tribvn, Chatillon, France) as previously described (28). Total collagen content was expressed as the ratio of tissue area stained with picrosirius red to total tissue surface. Pericellular collagen (i.e. the collagen specifically surrounding adipocytes) was expressed as the ratio of the sum of stained areas to the sum of field surfaces measured in 20 random fields examined at 10X magnification (18).

Immunomorphological analysis of adipose tissue

Immunohistochemical detection of total macrophages, defined as CD68+ cells (1:100, Dako cytomation, Trappes, France) and M2-macrophages, as CD163+ cells (1:100, AbD Serotec, Germany) were performed using the avidin-biotin peroxidase method (ABCYS Biospa, Milan, Italy) as previously described (29, 30). The staining was visualized using diaminobenzidine (DAB) (Dako cytomation), and slides were counterstained using haematoxylin. Digital slides of CD68+ cells were obtained by scanning total area at 20X magnification using a NanoZoomer Hamamatsu scanner (Hamamatsu Photonics KK, Systems Division) and digital slides of CD163+ cells were captured by scanning total area at 20X magnification using Zeiss Axio Imager 2 microscope (Carl Zeiss, Germany). Adipocytes and CD68+ cells were counted in 10 randomly chosen areas at 40X magnification using Calopix software (Tribvn). **Supplemental Figure 1** shows the specificity of CD68 staining. Adipocytes and CD163+ cells were counted in 10 randomly chosen areas using ZEN lite 2.3 Digital Imaging Software. The number of

186 macrophages was normalized for 100 adipocytes (expressed as percentage of macrophages) (29).
187 The number of crown-like structures (CLS), defined as at least three CD68+ cells surrounding an
188 adipocyte (31), was also counted for total tissue area. A score of fibro-inflammation was
189 calculated as the addition of CD68+ cell percentage and pericellular collagen percentage in each
190 fat compartment. To examine the effect of this score on metabolic variables, women were
191 subdivided in two subgroups with either a low or a high fibro-inflammation score according to
192 the median value of the distribution in each compartment.

193 ***Statistical analyses***

194 Student paired *t*-tests were computed to assess depot differences in total and pericellular collagen
195 percentage or macrophages percentage. Pearson correlation coefficients were computed to
196 examine associations between total collagen percentage, peri-adipocyte collagen percentage, age,
197 body fatness, body fat distribution, adipocyte sizes, CD68+ cell percentage, CD163+ cell
198 percentage, plasma inflammatory markers and cardiometabolic risk measurements. Spearman
199 correlation coefficients were computed to assess associations between VAT or SAT attenuation
200 and total collagen percentage or peri-adipocyte collagen percentage. Partial Pearson correlation
201 coefficients were computed to assess associations between pericellular collagen accumulation
202 and cardiometabolic risk factors after statistical adjustments for age, BMI or visceral adipose
203 tissue area. Adiposity measurements and metabolic outcomes were compared in women with low
204 versus high fibro-inflammation scores in each fat compartment using Student's t tests. Non-
205 normally distributed variables were log10- or Box-Cox-transformed. Statistical analyses were
206 performed using JMP software (SAS Institute, Carry, NC, USA).
207

RESULTS

Clinical characteristics of participants

The average age of the women was 47.2 years and they were slightly overweight with a mean BMI of 27.1 kg/m^2 (**Supplemental Table 1)**. They covered a wide range of adiposity value according to BMI values spanning from 20.3 to 41.1 kg/m^2. The average age of lean, overweight and obese women was similar (48.0±6.1, 46.5±5.7 and 47.2±5.5 years, respectively, *P*=0.66).

Regional differences in total and pericellular collagen percentage

There were regional variations in the distribution pattern of total collagen. In OM adipose tissue, collagen bundles were surrounding fat lobules (**Figure 1A**) whereas in the SC depot, total collagen fibers were arranged in bands of various thicknesses across the parenchyma (**Figure 1B**). Mean total collagen percentage was significantly higher in OM compared to SC adipose tissue (**Figure 1C**). There was no significant depot difference in peri-adipocyte collagen percentage (**Figure 1D and Figure 2**). Total collagen percentage in OM adipose tissue was positively and significantly associated with total collagen percentage in the SC depot (**Figure 1E**). A significant positive correlation was also observed between pericellular collagen percentage in OM adipose tissue and pericellular collagen percentage in SC adipose tissue (**Figure 1F**).

Total and pericellular collagen in relation with obesity, body fat distribution, adipose tissue attenuation and adipocyte size

Figure 2 shows representative accumulation of collagen around adipocytes in OM and SC adipose tissue samples from a lean woman and an obese woman. Obese women had higher peri-

adipocyte collagen percentage in both fat compartments compared to lean women (SC pericellular collagen percentage: 6.59±3.67 vs. 4.38±2.03%, *P*=0.03; OM pericellular collagen percentage: 6.32±2.71 vs. 4.54±2.35%, *P*=0.06).

Accordingly, body weight and BMI were positively associated with pericellular collagen accumulation in both adipose compartments (**Table 1**). Peri-adipocyte collagen percentage in OM adipose tissue was also positively and signicantly correlated with total body fat mass, waist circumference and abdominal adipose tissue areas. In the SC fat compartment, pericellular collagen was posivitely associated with SC adipose tissue area and tended to be correlated with total body fat mass, waist circumference as well as total and visceral adipose tissue areas. Negative associations were observed between visceral adipose tissue attenuation and peri-adipocyte collagen percentage in both adipose tissue compartments. There was no significant association between total collagen percentage in either adipose tissue compartment and measurements of body fatness and body fat distribution (**Table 1**). Total and pericellular collagen percentages in SC adipose tissue were negatively correlated with age. These associations did not reach significance in the OM fat compartment. Total collagen percentage in SC adipose tissue was negatively associated with SC adipocyte size. A similar association was observed in OM adipose tissue, but did not reach significance. A positive and significant relationship was found between adipocyte diameter and pericellular collagen accumulation in both depots.

Total and pericellular collagen in relation with cardiometabolic risk factors

Table 2 shows Pearson correlation coefficients between total or peri-adipocyte collagen in each adipose tissue depot and blood lipid profile, glucose homeostasis or plasma inflammatory markers. There was no significant association between total collagen percentage in either fat

compartment and any of the metabolic markers. On the contrary, higher pericellular collagen percentage in OM adipose tissue was associated with higher total-to-HDL-cholesterol ratio, fasting insulin and $HOMA_{ir}$ index as well as lower values of HDL-cholesterol. Positive trends were observed between peri-adipocyte collagen in the OM fat compartment and values of VLDL-cholesterol and triglycerides as well as plasma CRP levels. In SC adipose tissue, pericellular collagen accumulation was positively and significantly associated with plasma leptin concentrations and tended to be negatively associated with values of HDL-cholesterol (P=0.07) and plasma adiponectin concentrations (P=0.10).

Total and pericellular collagen in relation with macrophage infiltration

To examine the relationship between collagen accumulation and inflammation, OM and SC adipose tissue CD68+ cells (marker of total macrophage infiltration) were measured using immunohistochemistry in a subsample of 48 women exhibiting lean to moderately obese phenotype (**Figure 3**). **Figure 3A** shows a representative detection of a crown-like structure (CLS) composed of CD68+ macrophages (brown staining) in SC adipose tissue from an obese woman. In the whole sample, only 12 women had at least one CLS in SC adipose tissue and only 2 women had a least one CLS in OM adipose tissue. The number of CLS was very low in each positive sample (between 1 and 7 CLS per slide). All women with CLS were obese (n=8) or overweight (n=4) (mean BMI=31.0±3.2kg/m^2, mean waist circumference=100±5cm). Immunohistochemical staining of CD68+ cells was detected among collagen bundles (**Figure 3B**) and around adipocytes (**Figure 3C**). The number of infiltrating macrophages around adipocytes was counted and expressed as the number of CD68+cells per 100 adipocytes. No significant depot difference was observed for CD68+ cell percentage (OM: 16.62±8.63% vs. SC: 16.46±10.94% P=0.90, data not shown). Very strong correlations were found between

pericellular collagen percentage and CD68+ cell percentage in both fat compartments (**Figure 3 D-E**). These associations remained significant after adjustment for BMI or visceral adipose tissue area ($P<0.05$). In a similar manner, total collagen percentage was slightly but significantly associated with CD68+ cell percentage in the SC fat depot ($P=0.05$) (not shown). However, there was no significant association between total collagen percentage and CD68+ cell percentage in OM adipose tissue (not shown).

To examine the association between M2-macrophages and collagen accumulation, OM and SC adipose tissue CD163+ cells were measured using immunohistochemistry in a subgroup of 20 lean to obese women. No significant depot difference was observed for CD163+ cell percentage (OM: 9.42±6.21% vs. SC: 8.29±6.21% $P=0.39$, not shown). CD163+ cell percentage in SC adipose tissue was significantly related to pericellular collagen and total collagen in the SC fat depot (r=0.57, $P=0.008$; r=0.45, $P=0.05$, not shown). Only the association with pericellular collagen remained significant after adjustment for age, BMI and VAT ($P<0.03$). In the OM fat compartment, a positive association was observed between CD163+ cell and pericellular collagen percentage (r=0.45, $P=0.05$, not shown). Total collagen percentage was not associated with CD163+ cell percentage in OM adipose tissue ($P=0.33$, not shown).

As expected, CD68+ cell percentage in both fat compartments was positively and significantly related to body weight, waist circumference, BMI, total body fat mass, adipose tissue areas and adipocyte size ($P\leq0.002$ for all) (not shown). Higher CD68+ cell percentage in both fat compartments was also associated with higher values of VLDL-cholesterol and triglycerides as well as lower concentrations of HDL-cholesterol ($P\leq0.05$ for all). Similar associations were observed with CD163+ cell percentage in SC adipose tissue only ($P\leq0.05$). CD68+ cell percentage in SC adipose tissue was also positively correlated with fasting insulin and $HOMA_{ir}$

index (*P*=0.03 for both). In the OM fat compartment, these associations did not reach significance (not shown).

Fibro-inflammation marker score in relation with body fat distribution and cardiovascular risk factors

Considering that pericellular collagen and CD68+ cell amount were both significantly associated with adiposity values and risk variables, we created a fibro-inflammation marker in either OM or SC adipose tissue by combining their percentages in each depot.

In each fat compartment, women were subdivided in two subgroups with either low or high fibro-inflammation score according to the median value of the distribution. As expected, women with a high OM fibro-inflammation score (n=24) had significantly higher OM CD68+ cell percentage (**Figure 4A**) and OM pericellular collagen percentage (**Figure 4B**) compared to women with a low OM fibro-inflammation score (n=23). They had significantly higher CD163+ cell percentage (*P*=0.01, not shown). Women with a high OM fibro-inflammation score also had higher BMI (**Figure 4C**), VAT area (**Figure 4D**), SAT area (**Figure 4E**), $HOMA_{ir}$ index (**Figure 4G**), plasma triglyceride (**Figure 4H**) and CRP levels (not shown) as well as lower VAT attenuation (**Figure 4F**) and concentrations of HDL-cholesterol (**Figure 4I**) ($P \leq 0.05$ for all).

Very similar results were observed when women were stratified according to SC fibro-inflammation score. Women with high SC fibro-inflammation (n=24) had significantly higher SC CD68+ cell percentage, CD163+ cell percentage, SC pericellular collagen percentage, BMI, VAT area, SAT area, $HOMA_{ir}$ index, plasma triglyceride and CRP levels as well as lower VAT attenuation and HDL-cholesterol concentrations compared to women with a low SC fibro-inflammation marker value (n=24) ($P \leq 0.05$ for all, not shown).

DISCUSSION

The aim of the study was to examine if total and pericellular collagen accumulations are relevant for the pathophysiology of visceral obesity and related cardiometabolic risks. Using picrosirius red staining, we demonstrated that only pericellular collagen, especially in the OM fat compartment, was associated with higher BMI, body fat mass and adipose tissue areas as well as lower radiologic attenuation of VAT and altered cardiometabolic risk factors. Furthermore, we found positive associations between pericellular collagen percentage and total or M2-macrophages in both depots. By contrast, total collagen percentage in either adipose tissue compartment was not related to measurements of adiposity, body fat distribution or cardiometabolic risk, which raises questions about the relevance of the term "adipose tissue fibrosis" as used for other organs such as the liver. This is the first study to document in non-obese and obese women how OM and SC adipose tissue fibrosis, either measured with a whole-tissue or pericellular approach, relates to body composition and metabolic markers associated with visceral obesity.

In humans, the association between adipose tissue fibrosis and cardiometabolic risk factors remained unclear (14). Some studies reported (32-34) a positive relationship between whole tissue-based assessment of fibrosis (ex. mRNA expression of collagen subtypes or total red staining collagen accumulation) in SC adipose tissue and insulin resistance, while other studies found no significant association (18, 19, 22). One major finding of our study is to clearly show that within adipose tissue, the distribution of collagen (i.e. collagen around adipocytes), rather than the total amount of collagen, associates with altered metabolic phenotype. Total collagen accumulation may represent a physiological phenomenon within adipose tissue that supports tissue architecture and regulates physiological processes that occur during adipose tissue

remodeling (9, 16, 35). Its presence is possibly mediated by other factors than adiposity level. We suggest that whole tissue-based assessments of adipose tissue fibrosis are insufficient to reflect dysfunctional adipose tissue and altered metabolic risks. In agreement with this hypothesis, Abdennour et al. (28) demonstrated that the physical measures of adipose tissue stiffness were associated with metabolic variables, whereas the immunohistochemical quantification of total collagens was not.

Pericellular collagen accumulation, especially in OM adipose tissue, was associated with obesity level and CT-derived measurements. These results extend and support prior findings showing that picrosirius-red staining pericellular adipose fibrosis in both depots was significantly higher in morbidly obese compared to lean subjects (18). Pasarica et al. (19) demonstrated that COL6A3 mRNA expression in SC adipose tissue was positively associated with BMI and VAT mass. Considering that type VI collagen fibers were found only around adipocytes (18), the results of this study indirectly suggest that collagen deposition around cells increases with adiposity level. These findings are not unanimous as McCulloch et al. (22) recently demonstrated that OM and SC adipose tissue COL6A3 mRNA expression was not increased with BMI and metabolic alterations. According to our findings, only collagen deposition around adipocytes seems to increase with adiposity level in a population of lean to moderately obese subjects. Our results are consistent with the hypothesis that collagen deposition around the adipocyte, especially in OM fat compartment, may be more detrimental than total collagen accumulation and may contribute to adipose tissue dysfunction by limiting further adipocyte expansion (16, 18, 32). Additional studies are clearly needed to characterize the impact of such peri-adipocyte fibrosis on metabolic disease risk and adipose tissue expandability in humans.

Various patterns of total collagen deposition between adipose tissue depots were found in our samples of lean to obese women and in morbidly obese subjects (18). McCulloch et al (22) recently observed that COL6A3 mRNA expression was significantly higher in SC compared to OM adipose tissue. Inversely, we observed that total collagen percentage was significantly higher in OM compared to SC adipose tissue, whereas pericellular collagen percentage was similar between OM and SC adipose tissues. Differences among studies are possibly related to the method used to quantify collagen accumulation (messenger RNA expression of a subtype of collagen vs. red staining total or pericellular collagen percentage) and population characteristics or conditions (body weight stable or after body weight loss). Our finding that total collagen is higher in OM fat may be explained by the morphological and structural differences between abdominal fat depots. Indeed, collagen deposition is also observed around vessels (18) and OM adipose tissue is more vascularized than SC adipose tissue (36).

The present study also demonstrated strong correlations between pericellular collagen percentage and total macrophage percentage in both depots. These findings corroborate numerous data indicating a close relationship between adipose tissue fibrosis and immune cell accumulation (14, 18, 33, 37-39). The exact mechanisms to explain fibrosis accumulation in human adipose tissue are unknown. A few studies provided evidences that inflammatory cells might participate in the generation and degradation of adipose tissue ECM (33, 37, 38, 40). Spencer et al. (33) demonstrated that coculture of macrophages with adipocytes promoted alternatively activated, profibrotic macrophages (M2). By secreting profibrotic proteins such as TGF-β, M2 macrophages may be involved in the immune response to replace injured tissue by connective tissue (40). Consistent with these findings, our results show that M2-macrophages were related to pericellular collagen, suggesting a role of these immune cells in the generation of collagen

around adipocytes (33, 41). We also demonstrated that women with a high fibro-inflammation score were characterized by higher BMI, adipose tissue areas, plasma triglyceride levels, plasma CRP concentrations, $HOMA_{ir}$ index as well as lower VAT attenuation. Although our study was not designed to investigate the mechanisms underlying the association between pericellular collagen accumulation, macrophage infiltration and cardiometabolic risk profile, we propose that dynamic interactions between collagen deposition around adipocytes and macrophage infiltration may be detrimental, leading to tissue dysfunction and ectopic fat accumulation.

The notion that a high density of fat estimated by CT-derived measurements is a marker of adipose tissue fibrosis has been put forward in the literature (42). However, to our knowledge, this link has never been clearly established. Here we found that low radiologic density of adipose tissue in the OM compartment was related to higher peri-adipocyte collagen deposition, while total collagen accumulation in both depots was not correlated with radiologic attenuation measurements. We recently reported that CT-derived adipose tissue attenuation and area both contribute to explain variation in the cardiometabolic risk profile associated with the same biological parameter: visceral fat cell hypertrophy (43). Our results are entirely consistent with these findings. Significant correlations between adipocyte size and pericellular collagen accumulation were observed. VAT attenuation appears to be a marker of adipocyte hypertrophy (43), which is associated with CT adipose tissue areas and pericellular collagen accumulation, not adipose tissue fibrosis *per se*.

Limitations of the study should be acknowledged. These results are cross-sectional, which prevents us for concluding on cause-and-effect relationships. Our study only included women. Similar studies in lean-to-moderately obese men are difficult to perform. Considering that sex

have an effect on adipose tissue cellularity and that men are more likely to accumulate visceral adipose tissue (3), our results could be different in a sample of men.

In conclusion, as opposed to total collagen deposition, pericellular collagen accumulation, especially in the OM fat compartment, is related to total adiposity, adipose tissue areas, radiologic attenuation, total and M2 macrophage infiltration and cardiometabolic risk profile in lean to moderately obese women.

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CONFLICT OF INTEREST

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FIGURE LEGENDS

Figure 1: Representative images of total collagen staining (picrosirius red) in OM (**A**) and SC (**B**) adipose tissue samples from an overweight women. Percentage of total collagen staining (**C**) and pericellular collagen staining (**D**) in OM versus SC adipose tissue (n=53).* $P \leq 0.01$ (**E**) Correlation between total collagen percentage in the OM compartment and total collagen percentage in the SC compartment (n=53). (**F**) Correlation between pericellular collagen percentage in OM adipose tissue and pericellular collagen percentage in SC adipose tissue (n=53). Pearson correlation coefficients and *P* values are shown. Total collagen amount was expressed as the ratio of picrosirius red staining tissue area/total tissue area. Pericellular collagen quantification was determined by examining picrosirius red staining tissue area in 20 randomly chosen parenchyma fields at X10 magnification. OM=omental; SC=subcutaneous

Figure 2: Representative images at X10 magnification of pericellular collagen staining (picrosirius red) in OM and SC adipose tissue samples from a lean woman and an obese woman, selected according to their BMI value. OM=omental; SC=subcutaneous

Figure 3: (A) Representative detection of a crown-like structure (CLS) in SC adipose tissue from an obese woman. **(B)** Representative detection of CD68+ macrophages in collagen areas of SC adipose tissue from an obese woman. **(C)** Representative detection of immunohistochemical staining of CD68+ macrophages in SC adipose tissue from an obese woman. The arrows point to several examples of CD68+ cells revealed with DAB system (brown staining). Slides were counterstained with hematoxylin (blue staining). "m" indicates macrophages and "c" indicates collagen areas. Correlations between CD68+ cell percentage and pericellular collagen percentage in OM (**D**) or SC (**E**) adipose tissue. Pearson correlation coefficients and *P* value are shown. OM=omental (n=47); SC=subcutaneous (n=48)

573 **Figure 4**: OM fibro-inflammation marker score in relation to body fatness and metabolic
574 variables. Comparison of (**A**) OM CD68+ cell percentage, (**B**) OM pericellular collagen
575 percentage, (**C**) BMI, (**D**) VAT area, (**E**) SAT area, (**F**) VAT attenuation, (**G**) $HOMA_{ir}$ index,
576 (**H**) plasma triglyceride levels and (**I**) plasma HDL-cholesterol concentrations in women with a
577 low (n=23) or a high (n=24) OM fibro-inflammation marker score (which is the addition of OM
578 CD68+ cell percentage and OM pericellular percentage). Data are presented as mean ± SEM, **P*
579 ≤ 0.05

Figure 1

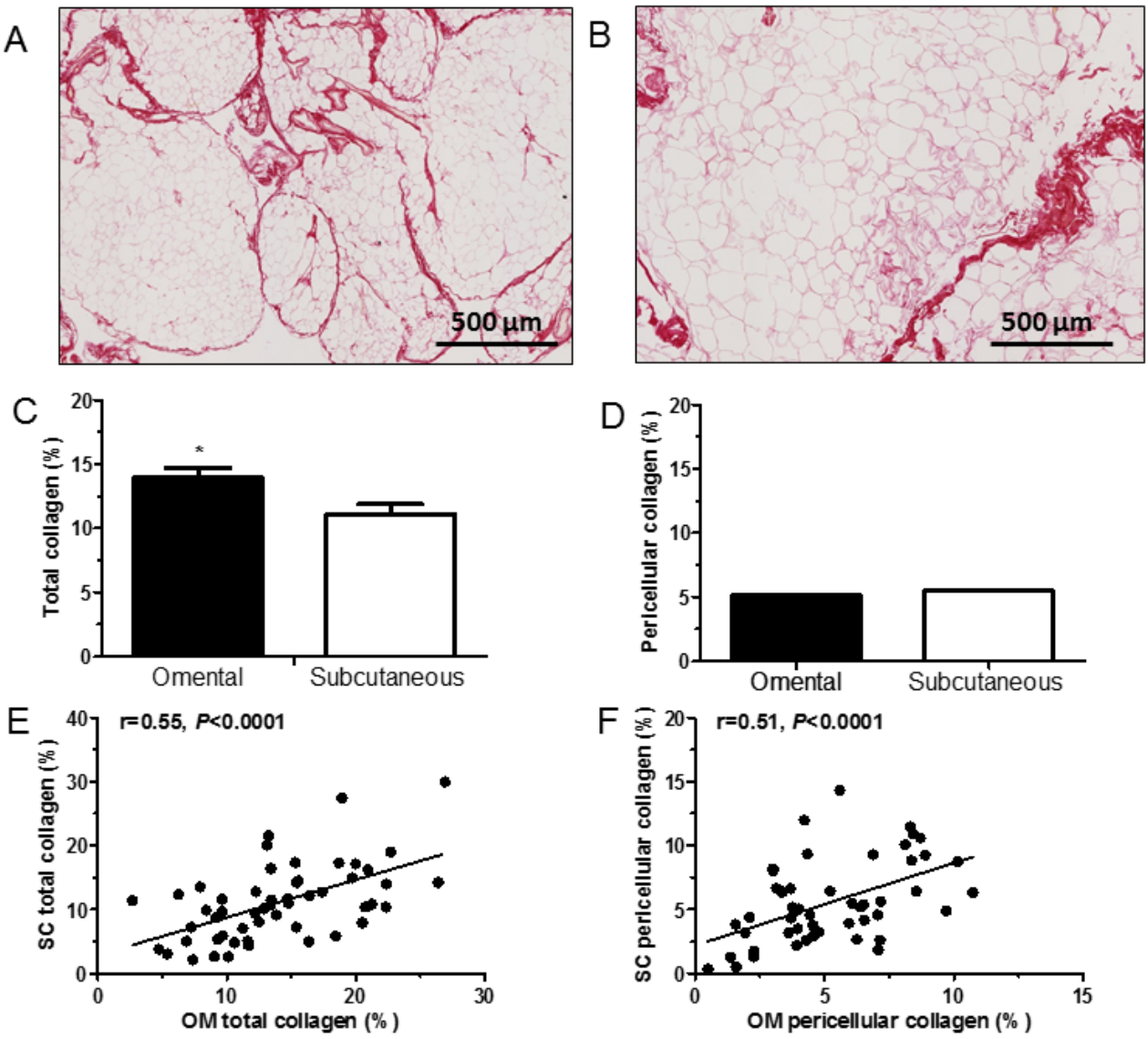

A
500 µm
B
500 µm
C
Total collagen (%)
*
Omental
Subcutaneous
D
Pericellular collagen (%)
Omental
Subcutaneous
E
r=0.55, P<0.0001
SC total collagen (%)
OM total collagen (%)
F
r=0.51, P<0.0001
SC pericellular collagen (%)
OM pericellular collagen (%)

Figure 2

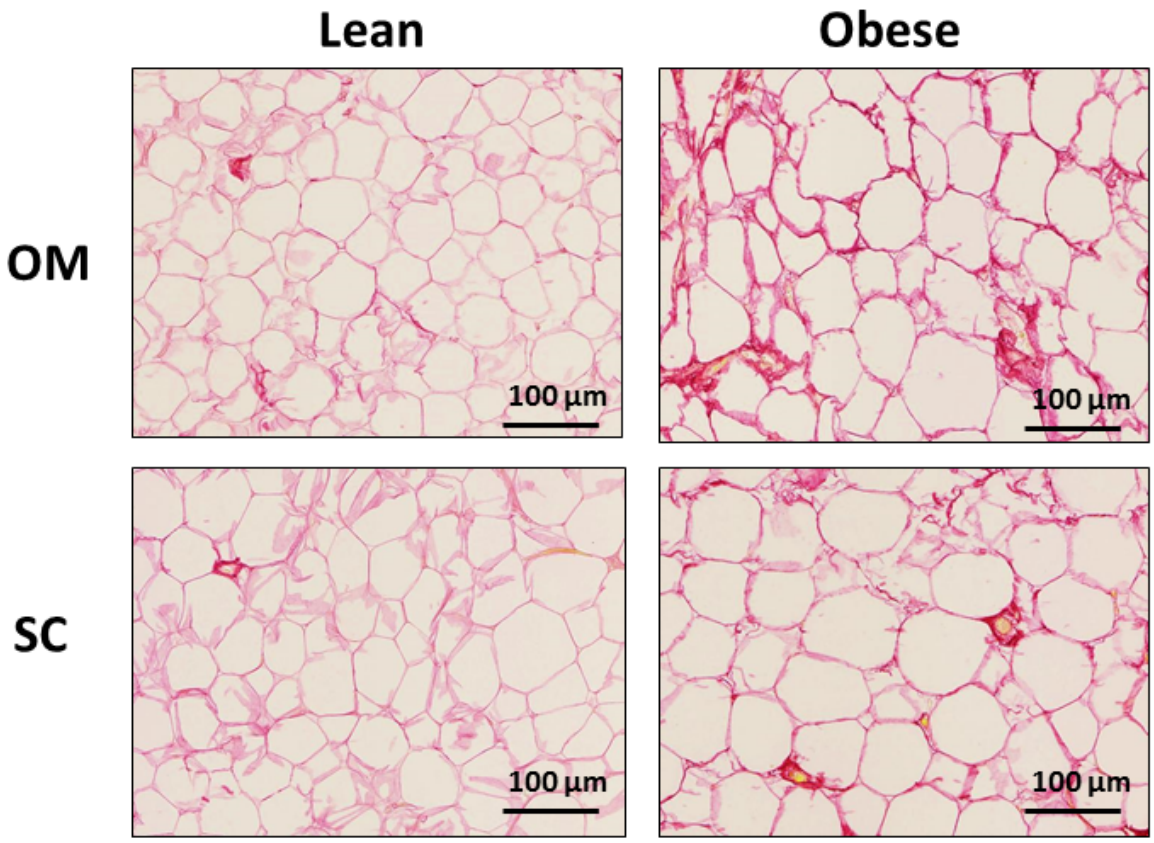


Figure 3

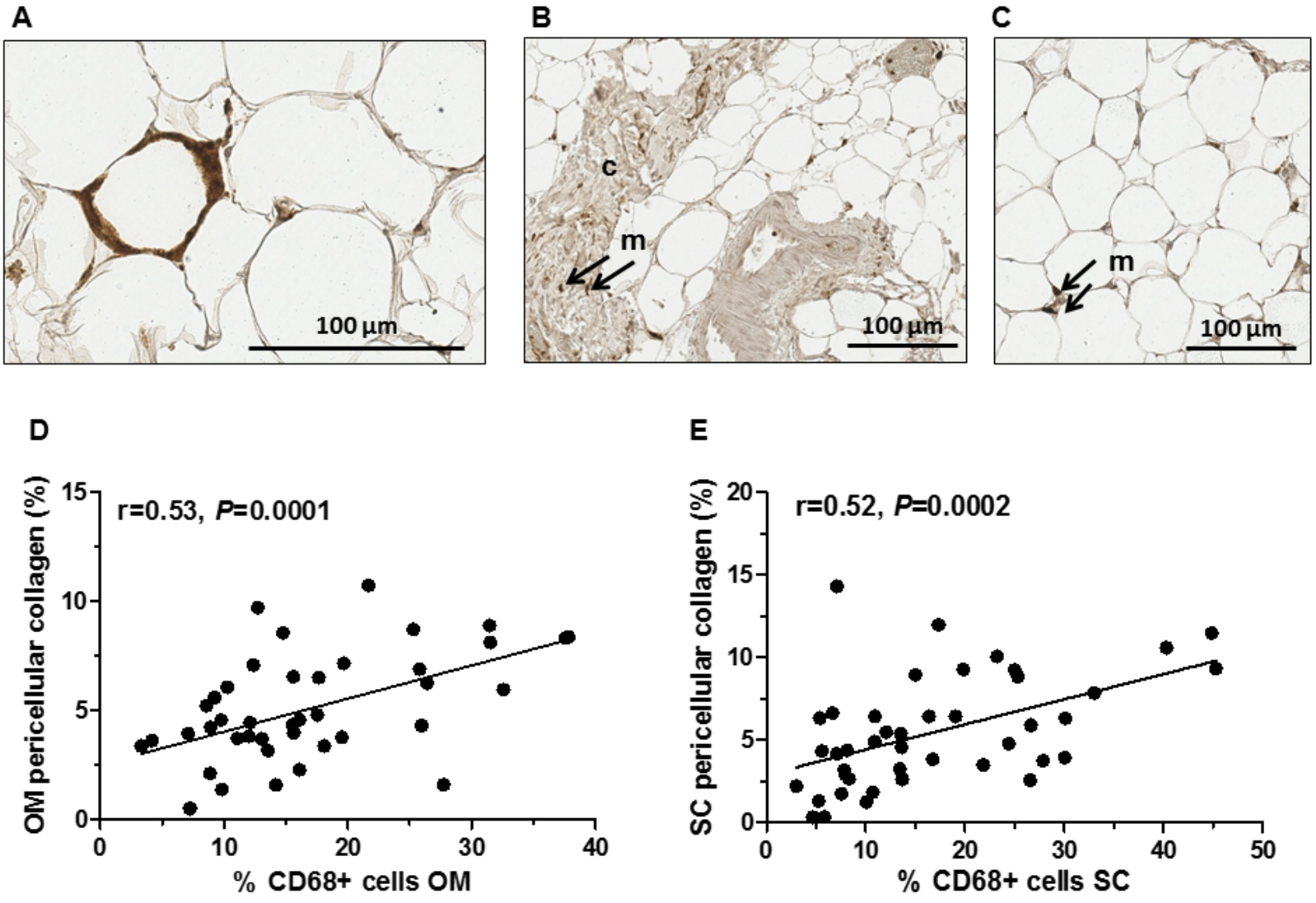
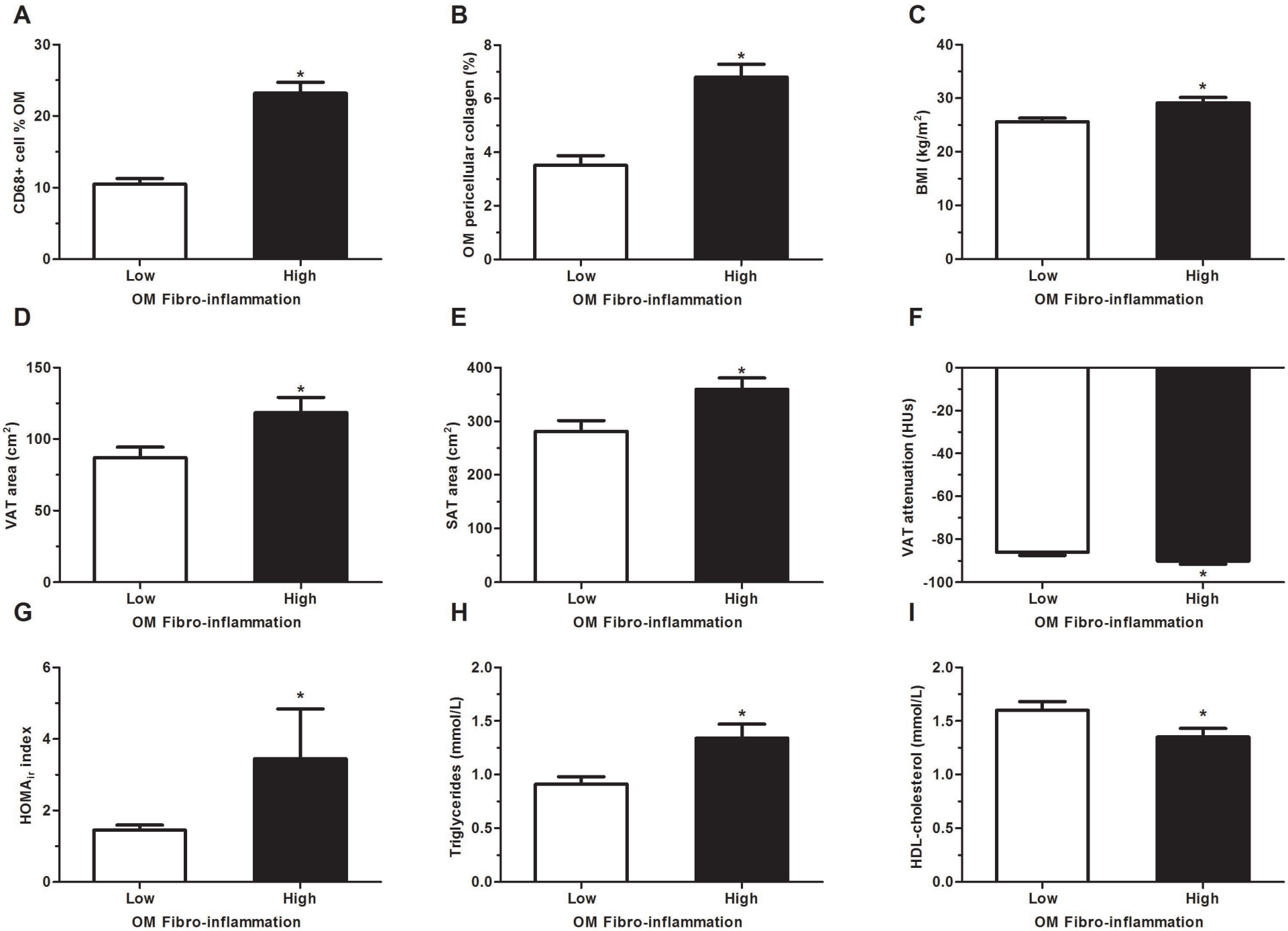

A
B
C
100 µm
c
m
100 µm
m
100 µm
D
r=0.53, P=0.0001
OM pericellular collagen (%)
% CD68+ cells OM
E
r=0.52, P=0.0002
SC pericellular collagen (%)
% CD68+ cells SC

Figure 4


A
CD68+ cell % OM
30
20
10
0
*
Low
High
OM Fibro-inflammation
B
OM pericellular collagen (%)
8
6
4
2
0
*
Low
High
OM Fibro-inflammation
C
BMI (kg/m2)
40
30
20
10
0
*
Low
High
OM Fibro-inflammation
D
VAT area (cm2)
150
100
50
0
*
Low
High
OM Fibro-inflammation
E
SAT area (cm2)
400
300
200
100
0
*
Low
High
OM Fibro-inflammation
F
VAT attenuation (HUs)
0
-20
-40
-60
-80
-100
*
Low
High
OM Fibro-inflammation
G
HOMAIr index
6
4
2
0
*
Low
High
OM Fibro-inflammation
H
Triglycerides (mmol/L)
2.0
1.5
1.0
0.5
0.0
*
Low
High
OM Fibro-inflammation
I
HDL-cholesterol (mmol/L)
2.0
1.5
1.0
0.5
0.0
*
Low
High
OM Fibro-inflammation