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ORIGINAL ARTICLE

Translational evidence of endothelial damage in obese individuals: inflammatory and prothrombotic responses

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Summary. *Background:* Obesity is associated with an increased atherothrombotic morbidity/mortality risk. However, there is no direct evidence of subclinical activation of the endothelium in obese subjects without other major cardiometabolic risk factors. *Objectives:* We applied a translational approach to investigate endothelial activation occurring in response to the components secreted by visceral and subcutaneous adipose tissue and their corresponding cell fractions obtained from obese subjects without other major cardiometabolic risk factors, as compared with non-obese controls. *Methods:* Fat pads and cell fractions were incubated with serum-free medium to obtain their secretomes, which were analyzed by protein arrays. Endothelial cells (ECs) were exposed to the different secretomes to evaluate changes in gene expression, composition and reactivity of the extracellular matrix (ECM), and cell growth and viability. *Results:* ECs incubated in the presence of obese secretomes displayed increased proliferation, altered cell morphology, augmented expression of VCAM-1, ICAM-1, and von Willebrand factor, and higher ECM reactivity towards circulating platelets. The visceral secretomes, especially the stromal one, induced the strongest expression of these markers, together with a more reactive ECM. These changes occurred through nuclear factor-κB (NF-κB) activation. *Conclusion:* This is the first translational study demonstrating that the cytokines secreted by the adipose tissue from obese individuals without other major cardiometabolic complications have a hazardous effect on the endothelium, through activation of the NF-κB pathway.

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Introduction

Obesity is a chronic systemic metainflammation that induces oxidative stress, endothelial dysfunction, and vasculopathy, and is an increased risk factor for atherothrombotic cardiovascular morbidity and mortality [1,2]. It is closely related to dyslipidemia, hypertension, insulin resistance, and diabetes mellitus, conditions that are known to damage the endothelium. There is evidence generated by studies in sera samples demonstrating that the changes in the adipose tissue of insulin-resistant obese individuals are related to the dysregulation of circulating proinflammatory and prothrombotic cytokines and adipokines [1,3,4]. However, there is still scattered evidence of the direct effects of the cytokines and adipokines secreted from different adipose tissue depots, taken from obese subjects, on endothelial cells (ECs).

Ongoing theories suggest that the initial event of adipose tissue dysfunction is induced by increases in both the number and size of adipocytes, resulting in hypoxia and defects in accumulation and storage of lipids. These events may occur in parallel with the infiltration of inflammatory macrophages activated by the release of chemoattractants, and/or with the switch of adipose tissue-resident macrophages to a proinflammatory phenotype caused by the altered adipocyte secretion, and/or with the conversion of preadipocytes to a functional macrophage phenotype. Moreover, recent studies reported the activation of obese adipose tissue-derived micro-ECs. Together, all of these phenomena lead to the activation of the non-adipocyte stromal cell fraction with the secretion of adipose tissue-derived factors, acting as chemokines and cytokines with a potential regulatory role in the development of chronic inflammation, angiogenesis, and atherothrombotic changes [5–9]. Several studies have investigated the regulation and secretion of cytoadipokines from different adipose tissue depots [5,10–12], although there are no studies on their direct impact on the endothelium.

The endothelium has the capacity to respond to local challenges to preserve its integrity. Depending on the origin and degree of the stimuli, the endothelial response may result in true endothelial dysfunction, an initial step in the development of the atherothrombotic process, and structural endothelial changes, with nuclear vacuolization, edema and cytoplasmic fragmentation, denudation, and lost of adhesion of ECs to their extracellular matrix (ECM) [13]. The higher amounts of endothelium-derived damage biomarkers in the circulation of obese individuals indicates that there is subclinical endothelial damage in obesity, which is an initial step in the development of atherothrombotic disease [1].

The present study was designed with two major goals: to elucidate the direct effect on the endothelium of the secretomes from visceral and subcutaneous adipose tissue obtained from obese subjects without other major cardiometabolic complications, in comparison with healthy controls; and to evaluate the relative contributions of the secretomes produced by either the adipocytes or the stromal non-adipocyte cell fractions of the adipose tissue. Experiments were designed to evaluate the potential proinflammatory, prothrombotic and proapoptotic effects of the different secretomes.

Materials and methods

Study participants

A total of 17 morbid obese subjects (body mass index [BMI] > 40 kg/m^2) from the Obesity Unit of the Hospital Clínic about to undergo gastric bypass surgery were included. The study was approved by the hospital's Ethics Committee. Written informed consent was obtained from all participants. Clinical history, physical data, blood pressure and electrocardiogram were registered. Selection criteria for low-risk obese individuals were partially based on the study of Karelis *et al.* [14]. Obese subjects who fulfilled the International Diabetes Federation (IDF) diagnostic criteria for metabolic syndrome (http://www.IDF.org) were excluded from the study. Twelve non-obese age-matched and sex-matched subjects (BMI: 21–26 kg m^{-2}) undergoing elective digestive surgery to correct benign conditions (vesicular lithiasis and eventrations) served as controls.

For both groups, participants with a family history of premature cardiovascular-related disease or deaths, a personal diagnosis of diabetes [15], dyslipidemia [3] or a clinical history/baseline electrocardiogram signs of cardiovascular disease and cardiovascular medication were excluded. Subjects receiving hormones (i.e. glucocorticoids, oral contraceptives, or other substitutive hormonal treatment) or non-steroidal anti-inflammatory drugs, immunosuppressive drugs or medications known to affect fat mass or metabolism (i.e. thiazolidinediones, modulators of adrenergic receptors, or lipid-lowering agents, including statins) and smokers were excluded from both groups. The 10-years obesity and BMI independent risk for atherothrombotic cardiovascular disease was estimated by calculating the Framingham score [16]. The main biochemical parameters were measured in serum, after an overnight fast. Serum insulin and adiponectin levels were measured with radioimmunoassays (RIAs). Insulin resistance was estimated by calculating the homeostasis model assessment of insulin resistance (HOMA-IR) index, with a threshold of 2.9, the reference value for the control population from our hospital.

Samples of adipose tissue biopsies: histological analysis

Visceral (omental) and subcutaneous (periumbilical) adipose tissue biopsy specimens (fat pads [FPs]) from morbid obese (n = 17) and non-obese (n = 12) individuals were obtained as paired samples during laparoscopic surgery, immediately transported to the laboratory, and used for subsequent cultures or fixed in paraformaldehyde. Fixed FPs were embedded in paraffin, sliced, and stained with hematoxylin/eosin, or immunostained with antibodies to CD45, for leukocytes, and to CD68, for macrophages. The total numbers of CD68-expressing and CD45-expressing cells were counted in 20 microscopic fields (n = 6). The percentages of CD68-positive and CD45-positive cells per 100 adipocytes for each sample were calculated.

Preparation of the conditioned media

All subsequent procedures were carried out under laminar airflow and sterile conditions. All cultures were performed in duplicate. Studies were performed by the protocol of Fain and Rodbell [17,18] with minor modifications. Each experimental replication involved tissue from a separate individual.

Subcutaneous and visceral adipose tissue was carefully cleaned, cut into small pieces (10 mg), incubated in Medium 199 (M199) at pH 7.4 for 2–5 min, and then centrifuged (400 ×g for 30 s) to reduce contamination with blood cells and pieces of tissue containing insufficient adipocytes to float. One part of the cleaned and cut tissue was kept as an intact FP and placed in M199 (pH 7.4) (1 g of FP per 5 mL). After 2 h, the medium in every dish was replaced, and incubation was continued for 24 h, at 37 °C, in the same non-fetal-serum containing medium to obtain the corresponding secretomes (non-obese visceral FP, non-obese subcutaneous FP, obese visceral FP, and obese subcutaneous FP). The remaining part was further processed to isolate the adipocyte and stromal cell fractions, as follows. One gram of FP was digested in 4 mL of M199 containing 1 mg/mL type II collagenase for 45–60 min, at 37 °C, under constant shaking. The reaction was stopped by dilution in phosphate-buffered saline (PBS), and the collagenase digest was then separated from the undigested tissue matrix by filtration on a silk mesh. A volume of 5 mL of medium was then added to the filtered digest, and, after centrifugation, isolated adipocytes were separated from the stromal–vascular fraction (400 ×g for 1 min) by flotation. The floating packed cells were washed twice with PBS. The stromal cell fraction was washed with an erythrocyte lysis buffer to reduce contamination. The adipocyte and the stromal cell

fractions were placed in 5 mL of M199 (pH 7.4), supplemented with antibiotics, at 37 °C in a humidified atmosphere containing 7% CO_2. After 2 h, the medium was replaced in every dish, and incubation was continued for 24 h, at 37 °C, in the same non-fetal-serum-containing medium to obtain the corresponding secretomes (visceral stromal, subcutaneous stromal, visceral adipocyte, and subcutaneous adipocyte). After 24 h of incubation, the conditioned media from the explants and cell fractions were stored at − 80 °C until being used.

Evaluation of cytokines in the conditioned media

Cytokines in the secretomes were analyzed with Human Cytokine Antibody Glass Arrays (Raybiotech Inc., Norcross GA, USA; G series 4000). Bioinformatic software was used for image analysis, background adjustment, and normalization of signal data. Differential non-parametric protein expression analysis was performed [19]. Differences in cytokines observed in the comparative study among groups were considered significant when $p < 0.01$. Cytokine network activation models were predicted with the Ingenuity Pathways Analysis tool (IPA). Additionally, adiponectin was quantified in the secretomes by RIA.

Experimental design for the in vitro *studies*

ECs obtained from human umbilical veins were exposed to the different secretomes ($n = 6$) to assess changes in: (i) cell morphology and proliferation; (ii) expression of VCAM-1 and ICAM-1; (iii) expression of von Willebrand factor (VWF) in the ECM; (iv) adhesion of platelets on the ECM generated by the ECs, under flow conditions; and (v) intracellular signal transduction mechanisms through p38 mitogen-activated protein kinase (MAPK), Erk42/44, and nuclear factor-κB (NF-κB).

Human EC culture: cell viability and morphology

ECs were isolated from human umbilical veins, and cultured as described previously [20]. ECs were exposed to the secretomes for 24 h for studies on cells. To evaluate the composition and reactivity of the ECM, EC monolayers were grown to confluence, on coverslips, with culture media containing 10% pooled human sera and 10% conditioned media for 7 days. Cells were extracted with EGTA to obtain the ECM. Changes in EC morphology and proliferation were evaluated by light microscopy in ECs grown under the conditions described, and with a bromodeoxyuridine (BrdU) Cell Proliferation Kit Assay, using luminescence.

Changes in the expression of adhesion receptors and VWF determined by immunogold labeling and real-time PCR

ECs and ECM on coverslips were fixed (4% paraformaldehyde in PBS) to detect VCAM-1 and ICAM-1 on ECs, and VWF on ECMs, as previously described [20].

Total RNA was extracted from ECs with TRIzol, and retrotranscripted with Superscript III, and real-time PCR was performed to quantify VCAM-1, ICAM-1, VWF and NF-κB gene expression, with SYBR Green fluorophor (primers are given in Table S1).

Platelet adhesion on the ECM: perfusion studies

ECM-coated coverslips were perfused through a parallel-plate perfusion chamber (800 s^{-1}, 5 min) [21] with citrated blood from healthy donors, fixed, and stained to measure the degree of platelet deposition on the perfused surface.

Evaluation of phosphorylation kinetics of p38 MAPK, Erk42/44, and IκB

Starving ECs were exposed to the secretomes for 30 s to 15 min and lysed. Proteins were resolved by 8% SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies to phosphorylated p38 MAPK and Erk42/44 [21]. Considering that translocation of NF-κB to the nucleus is preceded by the phosphorylation and degradation of IκB, we analyzed the phosphorylation of IκB in the presence of *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), which prevents its degradation by the 26S proteasome [22].

Statistical analysis

Results are expressed as mean ± standard error of the mean, including results from six different experiments in each experimental approach. Statistical analyses were performed with a two-tailed Student's *t*-test for paired samples, and results were considered to be significant when $P < 0.05$. When possible, experiments were evaluated blinded.

Results

Obese individuals under study are at low cardiometabolic risk

The baseline clinical and metabolic characteristics of study participants are shown in Table 1. The Framingham scale applied for the estimation of the 10-year obesity and BMI independent risk for cardiovascular disease classified all our obese subjects as low risk. Obese patients presented moderate insulin resistance, as estimated by HOMA-IR, and no alteration of glucose homeostasis.

Histological analysis of the adipose tissue reveals an altered adipose tissue structure

Obese adipose tissue showed a dystrophic structure with hypertrophic and irregular morphology of the adipocytes, and a higher presence of inflammatory infiltrate in both subcutaneous and visceral tissues, with the highest value in the obese visceral adipose tissue (Fig. 1). Percentages of

Table 1 Clinical and metabolic parameters of the subjects studied

	Obese subjects $N = 17$	Non-obese subjects $N = 12$
Age (years)	42.4 ± 10	46.4 ± 14
Sex (male/female)	2/15	2/10
BMI (kg/m^2)	44.3 ± 2.3	24.5 ± 2.6
Waist circumference (cm)	127.3 ± 4.01	83.9 ± 2.5
SBP/DBP (mmHg)	127.1/76.4 ± 2.8/1.7	115.1/71.5 ± 1.5/1.6
Fasting glucose (mmol/L)	5.5 ± 0.3	4.9 ± 0.5
2-h OGTT plasma glucose (mmol/L)	7.4 ± 1.6	–
Total cholesterol (mmol/L)	5.1 ± 1	4.3 ± 1.4
HDL/LDL (mmol/L)	1.2/3.3 ± 0.1/0.8	1.5/3 ± 0.2/0.6
Triglycerides (mmol/L)	1.4 ± 0.3	1 ± 0.3
Leukocytes (×10^9/L)	8.3 ± 1.6	6.4 ± 0.7
High-sensitivity CRP (mg/L)	13.3 ± 4.6	< 5*
HbA_{1c} (NGPS/DCC)	5.2 ± 0.3	4.0–5.5*
Insulin (pmol/L)	202.8 ± 78.5	15.3–98.6*
HOMA-IR	5.8 ± 1.8	< 2.93*
Adiponectin (μg/mL)	12.8 ± 3.5	16.7–23.3*

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; HOMA-IR (homeostasis model assessment of insulin resistance) (insulin mUI L^{-1} × glycemia (mM)/22.5); OGTT, oral 75-g glucose tolerance test; SBP, systolic blood pressure; Data are presented as mean ± standard error of the mean. *Laboratory age-matched and sex-matched normal parameter values.

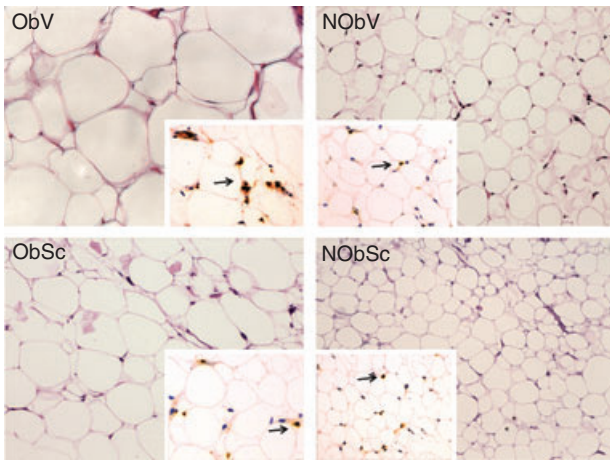

Fig. 1. Adipose tissue morphology. Micrographs (× 200) showing cell structure after hematoxylin/eosin staining in obese (Ob) and nonobese (NOb) visceral (V) and subcutaneous (Sc) adipose tissues. Inserts: Immunohistochemical detection of CD68-positive macrophages in the adipose tissue (indicated by arrows). Images are representative of six different subjects.

CD68-positive and CD45-positive cells were 14.8% ± 1.9% and 13.6% ± 2.5% in obese visceral tissue vs. 3.9% ± 0.5% and 3.3% ± 1% in non-obese visceral tissue ($P = 0.002$). Percentages of CD68-positive and CD45-positive cells were 8% ± 1% and 7.67% ± 1.5% in obese subcutaneous tissue vs. 2.2% ± 0.5% and 2% ± 0.7% in non-obese subcutaneous tissue ($P = 0.007$).

Non-obese and obese adipose tissues have different patterns of cytokine secretion

Secretomes obtained by incubating non-obese and obese FPs in serum-free medium for 24 h were analyzed by hybridization of protein arrays. Cytokines showing differences among groups with a statistical difference of $P < 0.01$ were selected. The differentially expressed cytokines are involved in inflammation, chemotaxis, proliferation, and hemostasis processes, and are associated with the Erk1/2, JAK, p38 MAPK and NF-κB pathways. Cytokine levels in the secretomes from obese FPs were generally higher, especially in those from obese visceral adipose tissue, than in those from non-obese FPs (Table 2). The most significant differences were observed for interleukin 6 (IL-6), monocyte chemotactic-1 (CCL-2/MCP-1), and monocyte chemotactic protein-2 (CCL-8/MCP-2), together with the chemokines CXCL1 (GRO), macrophage inflammatory protein-1β (CCL4/MIP-1β), and CCL14, angiogenic proteins such as angiogenin (ANG), hepatic growth factor (HGF), matrix metalloprotease (MMP9, MMP1), and tissue inhibitors of MMPs (TIMPs). These are central hubs in the network, and presented significant variations among all groups, with the highest levels being found in the obese visceral FP secretome (Fig. S1). Total adiponectin was generally reduced in the obese group (2.48 ± 0.48 vs. 4.12 ± 0.51 μg per 200 mg FP per mL conditioned medium, $P = 0.002$), with a lower but not statistically significant value being found in the obese visceral secretome (2.32 ± 0.26 vs. 2.68 ± 0.5 μg per 200 mg FP per mL conditioned medium, $P = 0.1$).

Obese adipose tissue secretome alters EC morphology and induces cell proliferation

ECs grown in the presence of the visceral and subcutaneous FP secretomes from non-obese donors formed a homogeneous monolayer, and cells exhibited normal sizes and shapes. In contrast, the secretomes from the obese visceral and subcutaneous FPs induced accelerated disorganized proliferation (micrographs; Fig. 2). These cells were irregular, detached easily, and released detritus to the culture media.

The rate of cells in division when exposed to the obese visceral and subcutaneous FP secretomes (24.2% ± 2.1% and 21% ± 0.4% of cells in division, respectively) was higher than in the presence of the corresponding non-obese secretomes (15.4% ± 0.9% and 15.3% ± 0.7% of cells in division, respectively, $P < 0.05$). BrdU incorporation confirmed these findings (bar diagrams; Fig. 2).

Obese adipose tissue secretome induces higher expression of adhesion molecules on ECs

VCAM-1 and ICAM-1 expression on ECs increased in response to the obese samples (micrographs; Fig. 3A). It was much more intense in the cell boundaries, especially in those areas of close proximity between cells. The densitometric

Table 2 Cytokine variations between obese visceral (ObV), obese subcutaneous (ObSc), non-obese visceral (NObV) and non-obese subcutaneous (NObSc) secretomes, quantified by protein arrays

ObV vs. NObV				ObSc vs. NObSc			
Protein ID	Fold change	Gene ID	$P < 0.01$	Protein ID	Fold change	Gene ID	$P < 0.01$
IL-6	48.75	*IL6*	0.00016	CCL14a	22.05	*CCL14*	0
MCP-2	48.32	*CCL8*	0.00016	MCP-1	15.84	*CCL2*	0.00433
CCL14a	42.45	*CCL14*	0	Angiogenin	14.96	*ANG*	0.00233
LYVE-1	27.83	*LYVE1*	0	LYVE-1	13.15	*LYVE1*	0.00023
GRO	23.93	*CXCL1*	0.00033	HGF	13.06	*HGF*	0.00066
MCP-1	21.16	*CCL2*	0.00583	CD14	12.69	*CD14*	0
MMP-9	17.02	*MMP9*	0.00018	MMP-9	11.91	*MMP9*	0.00037
Bate2 M	15.87	*B2M*	0.00186	MCP-2	9.12	*CCL8*	0.00712
MIP-1β	15.72	*CCL4*	0.002	Bate2 M	8.57	*B2M*	0.00581
TIMP-2	13.90	*TIMP2*	0.00451	Siglec-5	8.42	*SIGLEC5*	0.00111
CD14	13.77	*CD14*	0.00018	MIP-1β	7.93	*CCL4*	0.00266
HGF	13.55	*HGF*	0.00433	Nidogen-1	7.52	*NID1*	0.00837
Siglec-5	12.63	*SIGLEC5*	0.00018	Cathepsin S	7.13	*CTSS*	0.00465
Nidogen-1	11.91	*NID1*	0.00069	MMP-1	4.33	*MMP1*	0.00777
Platelet factor 4	11.34	*PF4*	0.00162	IL-17C	0.72	*IL17C*	0.00558
MMP-1	7.75	*MMP1*	0.00407	TECK	0.60	*CCL25*	0.00801
TIMP-4	6.44	*TIMP4*	0.00777	Oncostatin M	0.45	*OSM*	0.00633
IL-18Rβ	0.54	*IL18RAP*	0.00129	CKβ 8-1	0.28	*CCL23*	0.00016

HGF, hepatic growth factor; IL, inteleukin; MCP-1, monocyte chemotactic protein-1; MCP-2, monocyte chemotactic protein-2; MIP-1β, macrophage inflammatory protein-1β; MMP, matrix metalloprotease; TIMP, tissue inhibitor of matrix metalloprotease. Cytokine variations with $P < 0.01$ are presented as fold change. The predictive ingenuity network obtained pointed to inflammation, chemotaxis, angiogenesis and hemostasis-related cytokines as common central hubs (Fig. S1)

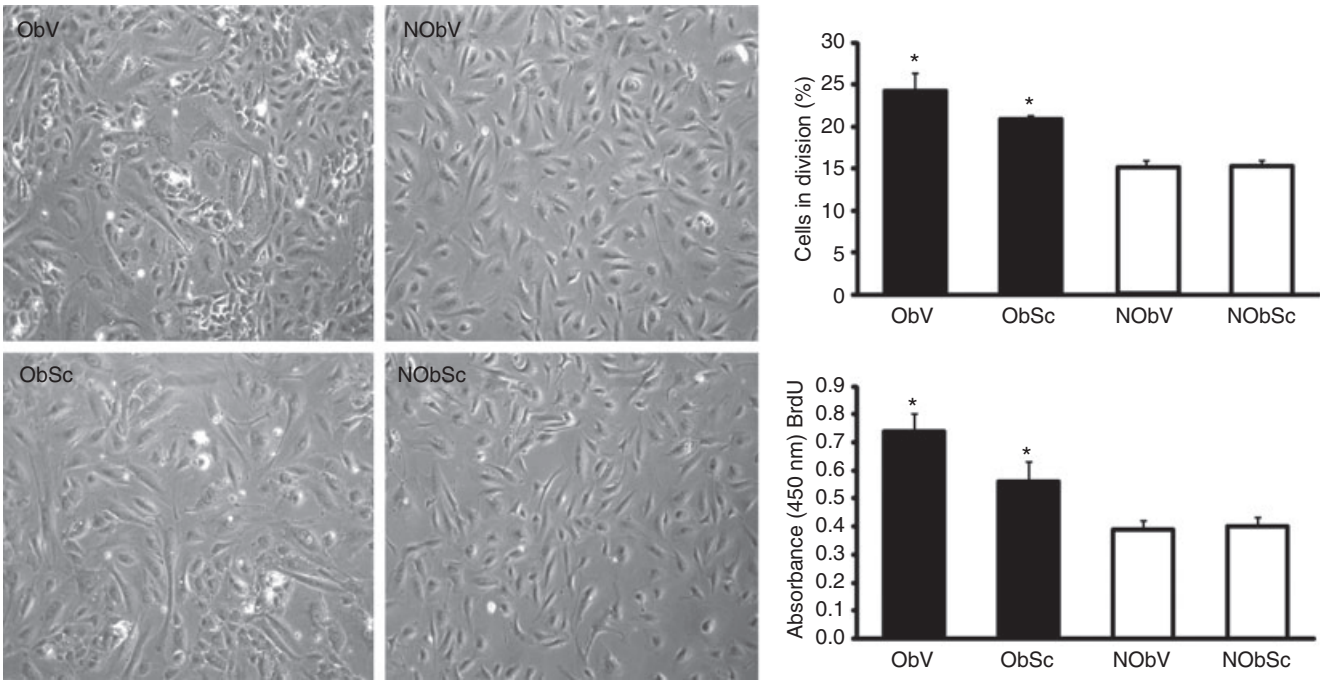

Fig. 2. Cell proliferation and morphological changes. Micrographs (× 320) show endothelial cell monolayers grown in the presence of obese visceral (ObV), obese subcutaneous (ObSc), non-obese visceral (NObV) and non-obese subcutaneous (NObSc) secretomes for 7 days. Bar diagrams show the percentages of cells in division (upper bar diagram, $n = 6$), and bromodeoxyuridine (BrdU) incorporation (lower panel, $n = 6$) in endothelial cells exposed to the same Ob/NOb secretomes. *$P < 0.05$ vs. the corresponding NOb value.

analysis indicated that the expression levels of VCAM-1 and of ICAM-1 were 142 ± 20 and 218 ± 42 pixels/μm^2, respectively, on ECs exposed to obese visceral FP secretomes ($P < 0.05$ for both results, as compared with 54 ± 18 and 60 ± 12 pixels/μm^2, respectively, on cells exposed to the corresponding non-obese products) and were 109 ± 19 and 133 ± 32 pixels/μm^2 on ECs exposed to obese subcutaneous FP secretomes ($P < 0.05$ for both results, as compared with 36 ± 1 and 48 ± 12 pixels/μm^2, respectively, on cells exposed to the corresponding non-obese secretomes). The obese visceral

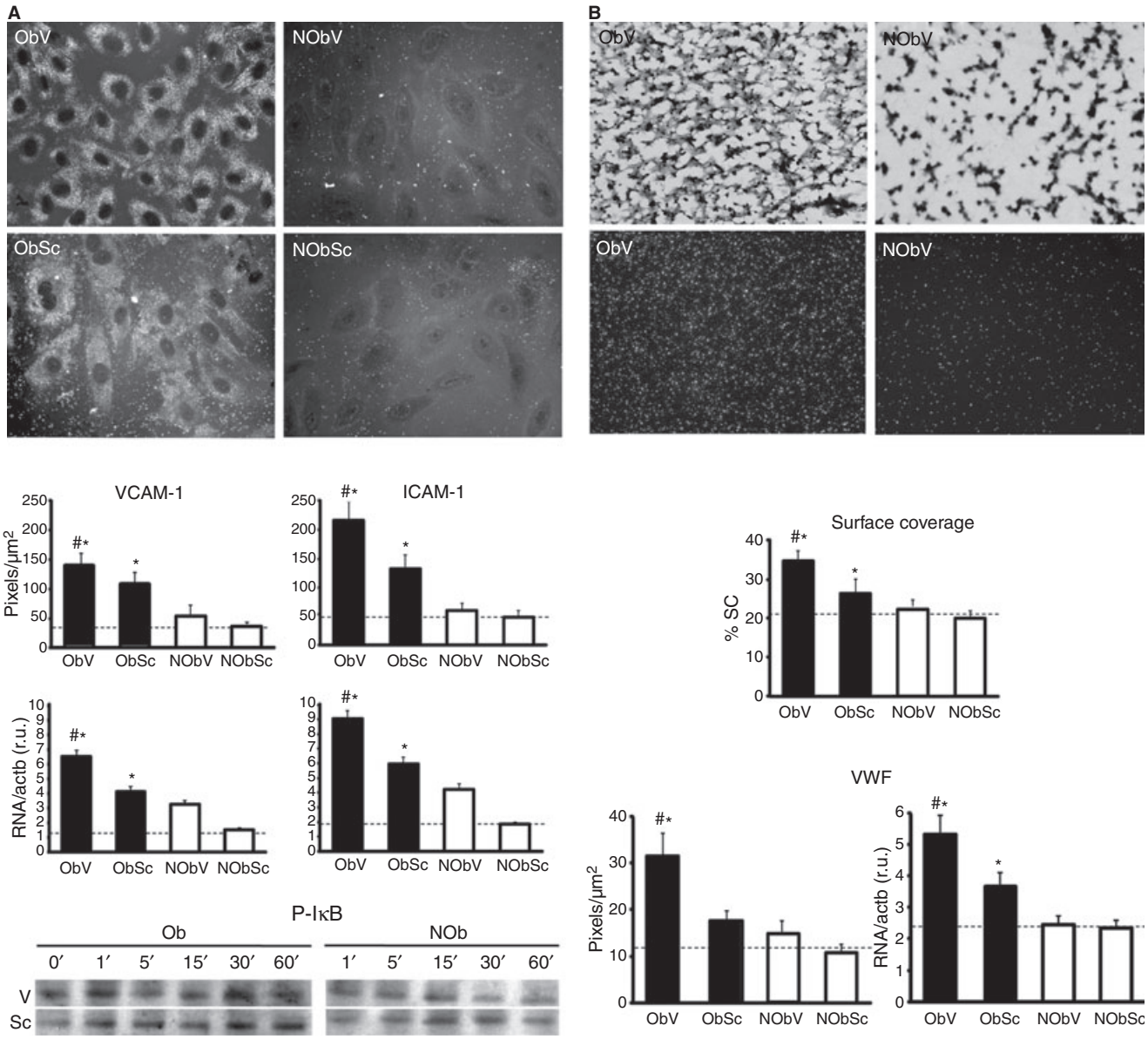

Fig. 3. Proinflammatory and prothrombotic effects of the obese secretomes on endothelial cells. (A) Expression of VCAM-1 and ICAM-1. Micrographs (× 320) obtained with the epipolarizing filter show cells exposed for 24 h to obese visceral (ObV), obese subcutaneous (ObSc), non-obese visceral (NObV), and non-obese subcutaneous (NObSc) secretomes. Bar diagrams represent expression of VCAM-1 and ICAM-1 proteins according to density of gold labeling (pixels/μm^2) (upper bar diagrams) and gene expression (relative units, r.u.) (lower bar diagrams). Dotted lines represent averaged data ($n = 12$) from control experiments carried out in the presence of regular culture media. Data are expressed as mean ± standard error of the mean (SEM) ($n = 6$). Western blots ($n = 6$) show the kinetics of IκB phosphorylation in endothelial cells exposed to the Ob and NOb secretomes. (B) Thrombogenicity of the extracellular matrix (ECM) generated. Micrographs (× 320) show platelet adhesion after perfusion of citrated blood (800 s^{-1}, 5 min) (upper micrographs), and von Willebrand factor (VWF) expression, detected by gold labeling (lower micrographs), on the ECM from cells grown in the presence of ObV, ObSc, NObV and NObSc secretomes for 7 days. Bar diagrams represent the surface covered by platelets (%SC) (upper bar diagram), and VWF protein (pixels/μm^2) and gene (relative units, r.u.) expression (lower bar diagrams). Dotted lines represent averaged data ($n = 12$) from control experiments carried out in the presence of regular culture media. Data are expressed as mean ± SEM ($n = 6$). *$P < 0.05$ in ObV vs. NObV, and $P < 0.05$ in ObV vs. ObSc.

secretome showed a much more proinflammatory effect ($P < 0.001$) than the obese subcutaneous one. The results are shown in Fig. 3A, in which a dotted line indicates the results in control experiments using ECs exposed to regular growth media (37 ± 6 and 49 ± 8 pixels/μm^2 for VCAM-1 and ICAM-1, respectively). These results were also confirmed by semiquantitative real-time PCR (bar diagrams; Fig. 3A).

Obese adipose tissue secretome induces activation of the NF-κB pathway in ECs

No differences were observed for NF-κB gene expression in response to any of the conditions assessed. However, western blot analysis showed that both the non-obese and the obese adipose tissue secretomes, from both the visceral and

subcutaneous locations, induced the phosphorylation of IκB, stabilized by the presence of ALLN, although to different degrees. When ECs were exposed to the obese secretomes, the activation of IκB was long-lasting (from 1 to 30 min) (Fig. 3A). Activation of IκB (measured by densitometry) was more intense in ECs exposed to the obese visceral secretomes, being increased 9.3 ± 1.3-fold, 2.7 ± 0.8-fold, 1 ± 0.5-fold, 4.4 ± 0.9-fold and 12 ± 2.1-fold vs. the corresponding non-obese secretomes after 1, 5, 15, 30 and 60 min of exposure. When ECs were exposed to the obese subcutaneous secretomes, phosphorylation of IκB increased 7.5 ± 1.0-fold, 2.2 ± 0.5-fold, 1.9 ± 0.2-fold, 2.3 ± 0.7-fold and 1.1 ± 0.8-fold at 1, 5, 15, 30 and 60 min. Phosphorylation of IκB in ECs exposed to the non-obese secretomes was weaker, being maximal at 15 min, after which time it decreased significantly.

Alteration of the reactivity and composition of the ECM generated by ECs

After perfusion of the ECM with citrated blood, the surface covered by platelets was significantly ($P < 0.05$) higher in the ECM generated by ECs grown in the presence of the obese secretomes (37% ± 2.8% and 28% ± 4% for the obese visceral and obese subcutaneous FPs, respectively) than in those generated after exposure to the non-obese secretomes (22% ± 1.7% and 20.2% ± 2.4% for the non-obese visceral and non-obese subcutaneous FPs, respectively), the obese visceral FPs being the most prothrombotic (Fig. 3B). These results are shown in Fig. 3B, in which a dotted line indicates the results in control experiments using ECs exposed to regular growth media (21.2% ± 1%).

In parallel with these results, the density of labeling for VWF was 32 ± 5, 18 ± 2, 15 ± 3 and 11 ± 1.8 pixels/μm^2 on the ECM generated by ECs grown in the presence of the secretomes from obese visceral FPs, obese subcutaneous FPs, non-obese visceral FPs, and non-obese subcutaneous FPs, respectively. Control values were 13.5 ± 0.8 pixels/μm^2. The results were also confirmed by real-time PCR (Fig. 3B).

Relative proinflammatory and prothrombotic effects of adipose tissue cell fractions: major role of the visceral stromal cell fraction

The relative proinflammatory effects of the different cell fractions (adipocytes and stroma) obtained from the different depots from the obese individuals were evaluated after exposing EC monolayers to their corresponding secretomes. All of the conditions evaluated increased the expression of the adhesion receptors analyzed (micrographs; Fig. 4A). The secretomes from the visceral cell fractions were the most proinflammatory, especially the stromal one ($P < 0.05$ vs. results obtained with the rest of the secretomes). These observations were confirmed by densitometric evaluation (expression of VCAM-1 and ICAM-1 of 173 ± 28 and 206 ± 16 pixels/μm^2, 115 ± 16 and 140 ± 16 pixels/μm^2, 132 ± 33 and 139 ± 16 pixels/μm^2, and 97 ± 22 and 98 ± 11 pixels/μm^2, in response to the visceral stromal, subcutaneous stromal, visceral adipocyte and subcutaneous adipocyte secretomes). The results were confirmed by quantitative real-time PCR (bar diagrams; Fig. 4A). In relation to these results, p38 MAPK became rapidly phosphorylated (at 1 min), the effect lasting at least until 15 min of exposure to the secretomes. After 5 min, the visceral stromal secretome induced the highest activation of p38 MAPK (Fig. 4A).

Microscopic observation revealed that exposure to the secretomes from the adipocyte fractions delayed EC growth. Evaluation of the activation of Erk42/44, which is a marker of proliferation, confirmed that these fractions did not induce proliferation at the same levels as the stromal ones. Phosphorylation of Erk42/44 in response to the stromal cell fraction was rapid (1 min), maintained for at least 5 min, and higher in the presence of the visceral stromal fraction (Fig. 4B).

The percentage of the surface covered by platelets on the perfused ECM from ECs exposed to the visceral stromal secretome (28.2% ± 2%) was significantly higher than that on the ECM from ECs exposed to the subcutaneous stromal secretome (21.2% ± 1.6%, $P < 0.05$) (Fig. 4B). Perfusion studies on the ECM from ECs exposed to the secretomes of the adipocyte cell fractions could not be performed, as layers of the ECM were removed together with ECs during the EGTA treatment, suggesting a defect in the quality of the ECM. In parallel with these results, the density of labeling for VWF (Fig. 4B) on the ECM generated by ECs exposed to the visceral stromal secretome (31 ± 2 pixels/μm^2) was significantly higher than that observed on the ECM from ECs exposed to the subcutaneous stromal secretome (18 ± 2 pixels/μm^2, $P < 0.05$). Changes at the gene expression level followed the same pattern.

Discussion

We have investigated the direct effect on the endothelium of adipose tissue secretomes from visceral and subcutaneous locations obtained from obese subjects with no other additional cardiometabolic risk factors than BMI and obesity itself. Previous data are only related to the deleterious effect of the serum from obese patients on ECs. To our knowledge, this is the first comprehensive study showing that the adipose tissue secretome from obese subjects induces activation and damage to ECs, as demonstrated by using of an established macrovascular experimental model. Moreover, these effects seem to be dependent on activation of the NF-κB pathway.

None of the obese participants included in the study could be diagnosed as suffering from metabolic syndrome, following the IDF consensus criteria (http://www.idf.org). However, signs of inflammation, such as increased high sensitive C reactive protein (hCRP) level, related insulin resistance, and decreased adiponectin level, were observed at the systemic level. In addition, applying the Framingham scale for estimating 10-year BMI and obesity independent cardiovascular risk, the

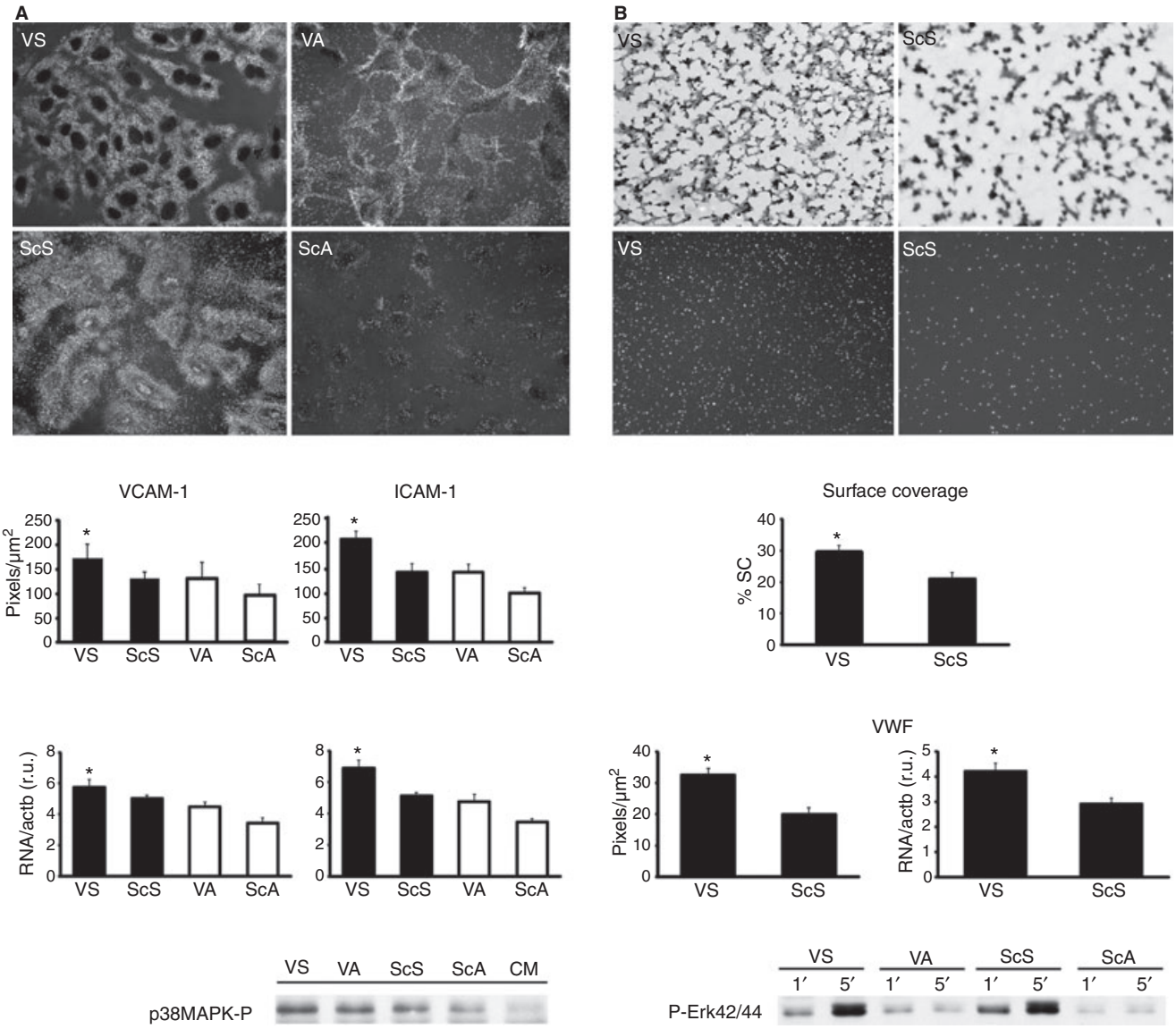

Fig. 4. Proinflammatory and prothrombotic effects of stromal and adipocyte obese secretomes on endothelial cells. (A) Expression of VCAM-1 and ICAM-1. Micrographs (× 320) obtained with the epipolarizing filter show endothelial cells exposed for 24 h to visceral stromal (VS), subcutaneous stromal (ScS), visceral adipocyte (VA) and subcutaneous adipocyte (ScA) secretomes. Bar diagrams represent the expression of VCAM-1 and ICAM-1 proteins according to density of gold labeling (pixels/μm^2) (upper bar diagrams) and gene expression (relative units, r.u.) (lower bar diagrams). Data are expressed as mean ± standard error of the mean (SEM) ($n = 6$). Western blot ($n = 6$) shows phosphorylation of p38 mitogen-activated protein kinase (MAPK) in endothelial cells exposed to the secretomes. (B) Thrombogenicity of the extracellular matrix (ECM). Micrographs (× 320) show platelet interaction, after perfusion of citrated blood (800 s^{-1}, 5 min) (upper micrographs), and von Willebrand factor (VWF) expression, as detected by gold labeling (lower micrographs), on the ECM from cells grown in the presence of VS and ScS secretomes for 7 days. Bar diagrams represent the surface covered by platelets (%SC) (upper bar diagram), and VWF protein (pixels/μm^2) and gene (relative units, r.u.) expression (lower bar diagrams). Data are expressed as mean ± SEM ($n = 6$). *$P < 0.05$ in obese visceral vs. obese subcutaneous stroma. Western blot ($n = 6$) show phosphorylation of Erk42/44 in endothelial cells exposed to the obese secretomes. CM: regular culture media.

obese individuals included in the present study were all classified as low-risk.

Levels of cytokines in the secretomes from obese adipose tissue were generally higher than in those from non-obese individuals, being notably higher in the visceral secretome. Bioinformatic analysis indicated that there were significant differences in the composition of the secretomes, depending on the tissue location, in both groups of subjects. The predicted network model pointed to several inflammatory, chemotaxis, proliferation and hemostasis-related cytokines as central common hubs.

In vitro exposure of macrovascular ECs to the obese secretomes increased proliferation, and induced both an inflammatory reaction and the release of an ECM more reactive to platelets. Cells became irregular in shape and size, and exhibit accelerated growth, as visualized by microscopy and confirmed by the activation of Erk42/44. Interestingly, these observations correlated with elevated levels of ANG and

HGF, both of which are involved in angiogenesis and proliferation. At the surface level, ECs exposed to the obese secretomes expressed significantly increased levels of the adhesion molecules VCAM-1 and ICAM-1, especially at gap junctions. This inflammatory reaction was paralleled by higher concentrations of proinflammatory cytokines, such as IL-6, and neutrophil and monocyte chemoattractants, such as MCP-1, MCP-2, MIP-1β, CXCL1, and CCL14, in the secretomes from obese adipose tissue than in those from non-obese adipose tissue [5,10]. As expected, because of its short half-life [23], no differences were observed in the levels of tumor necrosis factor-α (TNF-α) in the analyzed secretomes. However, our results for IL-6 reinforce previous findings by other authors showing increased secretion of visceral IL-6 in the portal vein, in correlation with increased levels of systemic major inflammatory cytokines, such as C-reactive protein, thereby providing a potential link between visceral fat and systemic inflammation [24]. In addition, the overexpressed chemokines MCP-1, MCP-2, MIP-1β, CXCL1 and CCL14 could be promoting the adhesion of leukocytes to the previously activated endothelium.

Using the same macrovascular EC in vitro model, we observed a prothrombotic phenotype in ECs in response to the obese adipose tissue secretomes. ECs produced an ECM that was more reactive towards platelets with a higher content of the adhesion protein VWF. This higher reactivity occurred in concert with elevated levels of MMP9 and MMP1 in the obese secretomes, and was particularly notable in response to the visceral secretome, which exhibited higher levels not only of the mentioned MMPs but also of the MMP inhibitors TIMP2 and TIMP4. Defects in the synthesis and breakdown of the ECM are seen as key processes in the development of atherosclerosis and its thrombotic complications. MMPs, which are endopeptidases that can degrade the ECM, promote macrophage invasion and angiogenesis, which seem to be involved in these processes. Therefore, MMPs may constitute potential biomarkers of disease [25]. In our experimental setting, although the levels of MMP9 and MMP1 were significantly higher in the obese media, the presence of the specific inhibitors TIMP2 and TIMP4 in the obese visceral media could explain the higher integrity with marked reactivity of the produced ECM.

Moreover, our results indicate that obese adipose tissue secretes decreased levels of adiponectin along with increased levels of hazardous cytoadipokines. In arteries, adiponectin has been associated with a reduction of the intima–media thickness and the attenuation of inflammatory endothelial activation by inhibiting the activation of NF-κB, reducing the TNF-α-stimulated secretion of adhesion molecules, and inhibiting foam cell formation, as well as platelet aggregation and T-cell recruitment and accumulation [26,27]. We have demonstrated clear damage to ECs exposed to the secretomes from the adipose tissue of obese individuals, especially those from the visceral location, which occurs through the activation of signaling pathways, including p38 MAPK and Erk42/44, ending in activation of the transcription factor NF-κB. These observations are in accordance with those in a previous report [28]. Our results, taken together, sustain the hypothesis that there is a connection between the decreased levels of adiponectin and the upregulation of the inflammatory pathway through activation of specific signaling cascades that may alter transcriptional mechanisms [29].

Furthermore, regarding the influence of each cell fraction, we have demonstrated that the hazardous components derived from the visceral stroma cause greater damage to the endothelium. We have therefore provided evidence reinforcing recent hypotheses [6,8,9] that propose the activation of an inflammatory cell phenotype in the heterogeneous visceral stromal cell fraction of obese adipose tissue as one of the driving forces in subclinical endothelial activation.

Intra-abdominal accumulation of fat is believed to be the generator of the obesity-associated proinflammatory state, and it is considered to be a strong and independent predictor of adverse atherothrombotic outcomes [11]. Interestingly, our present results indicate that, although the visceral adipose tissue has the most deleterious potential, subcutaneous adipose tissue is also capable of secreting deleterious components, contributing to the maintenance of the inflammatory environment on the endothelium.

Our experimental observations suggest that those cytokines secreted by the adipose tissue of obese subjects, without any cardiometabolic risk factor other than BMI, have a direct persistent adverse effect on the endothelium. The present study covers an important gap in our understanding of the functional properties of the cytokines produced by the obese adipose tissue. In our understanding, once excessive fat accumulation overwhelms the regional expandability limit, local factors induce the activation of the stromal cell fraction. This event leads to a sustained alteration in the adipose tissue secretion pattern. Systemic macrovascular endothelial activation in obesity is the consequence of the synergic proinflammatory and thrombogenic profile of the obese secretome. To what extent one or more cytokines have a more prevalent effect remains a subject that is open to further research.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Comparative proteome network assembled by Ingenuity Pathways Analysis.
Table S1. List of primers used for real-time PCR analysis.

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