Increased adipose tissue expression of lipocalin-2 in obesity is related to inflammation and matrix metalloproteinase-2 and metalloproteinase-9 activities in humans

V. Catalán & J. Gómez-Ambrosi & A. Rodríguez &
B. Ramírez & C. Silva & F. Rotellar & M. J. Gil &
J. A. Cienfuegos & J. Salvador & G. Frühbeck

Abstract: Lipocalin-2 (LCN2) is a novel adipokine with potential roles in obesity, insulin resistance, and inflammation. The aim of the present work was to evaluate the effect of obesity on circulating concentrations and gene and protein expression levels of LCN2 in human visceral adipose tissue (VAT) as well as its involvement in inflammation. VAT biopsies from 47 subjects were used in the study. Real-time PCR and Western-blot analyses were performed to quantify levels of LCN2 in VAT as well as the association with other genes implicated in inflammatory pathways. Forty-four serum samples were used to analyze the circulating concentrations of LCN2. Zymography analysis was used to determine the activity of matrix metalloproteinase (MMP) in VAT. Obese patients exhibited increased mRNA ($p<0.0001$) and protein ($p=0.017$) expression levels of LCN2 compared to lean subjects. Although no differences in plasma LCN2 concentrations were observed, increased circulating LCN2/MMP-9 complex levels were found ($p=0.038$) in the obese group. Moreover, obese individuals showed increased ($p<0.01$) activity of MMP-2 and MMP-9/LCN2 complex, while a positive correlation ($p<0.01$) between MMP-2 and MMP-9 activities and BMI was observed. Gene and protein expression levels of LCN2 in VAT were positively associated with inflammatory markers ($p<0.01$). These findings represent the first observation that mRNA and protein levels of LCN2 are increased in human VAT of obese subjects. Furthermore, LCN2 is associated with MMP-2 and MMP-9 activities as well as with proinflammatory markers suggesting its potential involvement in the low-grade chronic inflammation accompanying obesity.

Keywords: Lipocalin-2 . Obesity. Gene expression . Visceral adipose tissue . Inflammation

V. Catalán : J. Gómez-Ambrosi : A. Rodríguez: B. Ramírez :
G. Frühbeck
Metabolic Research Laboratory, Clínica Universitaria de Navarra,
University of Navarra,
Pamplona, Spain

C. Silva: J. Salvador: G. Frühbeck (M)
Department of Endocrinology, Clínica Universitaria de Navarra,
University of Navarra,
Avda. Pío XII, 36,
Pamplona 31008, Spain
e-mail: gfruhbeck@unav.es

F. Rotellar : J. A. Cienfuegos
Department of Surgery, Clínica Universitaria de Navarra, University of Navarra,
Pamplona, Spain

M. J. Gil
Department of Biochemistry, Clínica Universitaria de Navarra, University of Navarra,
Pamplona, Spain

V. Catalán : J. Gómez-Ambrosi : A. Rodríguez: B. Ramírez :
C. Silva: F. Rotellar : M. J. Gil: J. A. Cienfuegos : J. Salvador:
G. Frühbeck
CIBER Fisiopatología de la Obesidad y Nutrición http://www.ciberobn.es/

INTRODUCTION

Adipose tissue plays an active role in energy balance by regulating lipid storage [1] as well as in metabolic homeostasis via the secretion of a wide range of signaling proteins collectively called adipokines [2–4], which are directly involved in the pathogenesis of obesity-related derangements such as insulin resistance, cardiovascular diseases, and inflammation [5–7].

Lipocalin-2 (LCN2), also known as neutrophil gelatinaseassociated lipocalin and 24p3, is a 25 kDa secretory glycoprotein member of the highly heterogeneous family of lipocalins. Besides its function as a carrier protein for small hydrophobic molecules including iron, LCN2 is a component of the innate immune system with a key role in the acute-phase response to infection and in the induction of apoptosis [8, 9]. It was first discovered in specific granules of human neutrophils, but it is also expressed in many tissues including adipose tissue, liver, kidneys, and lungs [10, 11]. Recent reports have described this protein as an adipokine closely related to obesity and insulin resistance [12, 13]. Gene and protein expression levels of LCN2 in liver and adipose tissue as well as circulating concentrations are increased in db/db obese diabetic mice compared with their lean littermates [12–14]. Furthermore, higher circulating levels of LCN2 have been described in obese humans being associated with anthropometric variables and measures of insulin resistance [12]. LCN2 expression is induced by dexamethasone and tumor necrosis factor (TNF)-a in 3T3-L1 adipocytes [13], while circulating concentrations are reduced in response to the peroxisome proliferator-activated receptor y (PPARy) agonist rosiglitazone in both mice and humans [12]. Recently, the upregulation of LCN2 by insulin in omental adipose tissue explants and its increased circulating levels after hyperinsulinemic induction in humans have been described [15].

Lipopolysaccharide (LPS) strongly stimulates LCN2 expression in blood cells [16] as well as in adipose tissue and liver [17] strengthening its role as an acute-phase reactant. In this sense, it has been shown that LCN2 regulates the inflammatory response during ischemia and reperfusion of the transplanted heart [18]. Furthermore, the expression of LCN2 in adipocytes was strongly induced by the proinflammatory cytokine interleukin 1-β [19]. Since obesity is considered a state of low-grade chronic inflammation [20, 21], the implication of LCN2 in the inflammatory signaling pathways activated in obesity seems plausible.

In addition, an increased expression of matrix-metabolizing enzymes is observed in obesity development at the same time as being a hallmark of many inflammatory processes. The family of matrix metalloproteinases (MMPs) guarantees the balance between synthesis and degradation of matrix proteins [22]. Human adipose tissue releases MMP2 and MMP-9 during adipocyte differentiation suggesting that the modulation of the extracellular components through the production of MMPs might exert a pivotal role in the remodeling that occurs during the development of obesity [23]. In this regard, LCN2 also exits as a covalent heterodimer of 125 kDa together with MMP-9, modulating MMP-9 activity by protecting it from degradation [24] implying that LCN2 might be, thus, indirectly involved in adipogenesis or obesity development.

In this study, we analyzed the influence of obesity on both gene and protein expressions of LCN2 in human visceral adipose tissue as well as its circulating concentrations. To test the hypothesis that LCN2 operates as a modulator of inflammation in obesity, we studied the relation of LCN2 with key genes and circulating proteins involved in inflammation in a well-characterized group of obese patients comparing it with lean volunteers. We also assessed the possible relevance of the LCN2/MMP-9 complex in obesity.

METHODS

Patient selection

Forty-seven samples of omental adipose tissue of eight lean (LN) and 39 obese (OB) volunteers were used to analyze the effects of obesity on LCN2 as well as on the gene expression levels of other related inflammatory markers such as TNF-α, MMP-2, MMP-9, osteopontin (OPN), and macrophage antigen CD68 (CD68). Volunteers for the study were recruited among patients attending the Departments of Endocrinology and Surgery of the Clínica Universitaria de Navarra and were classified as obese according to both body mass index (BMI > 30 kg/m^2) and whole body fat percentage (BF > 25% for males and BF > 35% for females). BMI was calculated as weight in kilograms divided by the square of height in meters and body fat was estimated by air-displacementplethysmography (Bod-Pod®, Life Measurements, Concord, CA, USA) [25]. Protein expression levels of LCN2 and MMP activity in visceral adipose tissue (VAT) were assessed in a subgroup of 20 morbidly obese and five lean volunteers. In addition, a group of 13 lean volunteers and 27 obese patients was used to analyze the effect of obesity on the circulating concentrations of LCN2 as well as the LCN2/MMP-9 complex levels.

The samples were collected from patients undergoing either Nissen fundoplication (for hiatus hernia repair in LN volunteers) or Roux-en-Y gastric bypass (RYGB; for obesity treatment in OB subjects) at the Clínica Universitaria de Navarra. Both interventions were carried out via a laparoscopic approach. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

The study was approved, from an ethical and a scientific standpoint, by the hospital's ethical committee responsible for research, and the written informed consent of participants was obtained.

Blood assays

Plasma samples were obtained by venipuncture after an overnight fast. Glucose was analyzed based on enzymatic spectrophotometric reactions by an automated analyzer (Hitachi Modular P800, Roche, Basel, Switzerland). Insulin was measured by means of an enzyme-amplified chemiluminescence assay (IMMULITE®, Diagnostic Products Corp., Los Angeles, CA, USA). Insulin resistance and sensitivity were calculated using the HOMA and QUICKI indices, respectively. Total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol levels were calculated as previously described [26]. Uric acid, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and y-glutamyltransferase were measured by enzymatic tests (Roche) in an automated analyzer (Roche/Hitachi Modular P800). High sensitivity C-reactive protein (CRP), fibrinogen and von Willebrand factor antigen (vWF) concentrations were determined as previously described [26]. Leptin was measured by a double-antibody RIA method (Linco Research, Inc., St. Charles, MO, USA). Intra- and inter-assay coefficients of variation were 5.0% and 4.5%, respectively. LCN2 and LCN2/MMP-9 complex levels were assessed using commercially available ELISA kits (R&D Systems Europe Ltd., Abingdon, UK) according to the manufacturer's instructions with intra- and inter-assay coefficients of variation of 3.7% and 6.5 %, respectively, for the former and 3.1% and 6.4% for the latter.

RNA extraction and real-time PCR

RNA isolation from adipose tissue was performed by homogenization with an ULTRA-TURRAX® T 25 basic (IKA Werke GmbH, Staufen, Germany) using QIAzol® Reagent (Qiagen, Valencia, CA,

USA). Samples were purified with the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's directions and treated with DNase I (RNase-free DNase Set, Qiagen) in order to remove any trace of genomic DNA. For first strand cDNA synthesis constant amounts of 2 μg of total RNA were reverse transcribed in a 40 μl final volume using random hexamers (Roche) as primers and 400 units of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) [27].

The transcript levels for LCN2, CD68, MMP-2, MMP-9, OPN, and TNF-α were quantified by Real-Time PCR (7300 Real Time PCR System, Applied Biosystem, Foster City, CA, USA). Primers and probes (Online Supplemental Table 1) were designed using the software Primer Express 2.0 (Applied Biosystems) and purchased from Genosys (Sigma, Madrid, Spain). Primers or TaqMan® probes encompassing fragments of the areas from the extremes of two exons were designed to ensure the detection of the corresponding transcript avoiding genomic DNA amplification. The cDNA was amplified at the following conditions: 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 59°C, using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The primer and probe concentrations for gene amplification were 300 and 200 nmol/l, respectively. All results were normalized to the levels of the ribosomal 18S rRNA (Applied Biosystems) and relative quantification was calculated using the AACt formula [28]. Relative mRNA expression was expressed as fold expression over the calibrator sample (average of gene expression corresponding to the LN group) as previously described [29]. All samples were run in triplicate and the average values were calculated.

Western blot studies

Tissues were homogenized, and protein content was measured as previously described [30]. Equal amounts of protein (25 μg) were run out in 12% SDS-PAGE, subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked in Tris-buffered saline (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.0) with 0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk for 1 h at room temperature (RT). Blots were then incubated overnight at 4°C with a goat polyclonal anti-LCN2 antibody (Abcam, Cambridge, UK) or murine monoclonal anti-P actin (Sigma). The antigenantibody complexes were visualized using horseradish peroxidase-conjugated anti-goat or anti-mouse IgG antibodies (1:5,000) and the enhanced chemiluminescence ECL detection system (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was determined by densitometric analysis with the Gel Doc™ gel documentation system and Quantity One 4.5.0 software (Bio-Rad) and normalized with P-actin densitometric values. All assays were performed in duplicate.

Immunohistochemistry of LCN2 and CD68

The immunohistochemistry of LCN2 and CD68 was carried out using the indirect immunoperoxidase method. Sections (6 μm) of formalin-fixed paraffin-embedded VAT were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol, and treated with 3% $_{H2O2}$ (Sigma) in absolute methanol for 10 min at RT to quench endogenous peroxidase activity. Then, slides were blocked during 1 h with 1% goat or murine serum (Sigma) diluted in Trisbuffered saline (TBS; 50 mmol/l Tris, 0.5 mol/l NaCl; pH 7.36) to prevent nonspecific adsorption. Sections were incubated overnight at 4°C with goat polyclonal anti-human LCN2 (Abcam) antibody diluted 1:50 in TBS or mouse monoclonal anti-human CD68 antibody (DakoCytomation, Glostrup, Denmark) diluted 1:100 in TBS. After three washes (5 min each) with TBS, sections were incubated with horseradish peroxidase-conjugated anti-goat (Zymed, San Francisco, CA, USA) or anti-mouse (Amersham Biosciences) polyclonal IgG diluted 1:100 in TBS for 1 h at RT. After washing in TBS, peroxidase reaction was visualized with a 3,3′-diaminobenzidine (DAB, Amersham Biosciences)/H2O2 solution (0.5 mg/ml DAB,

0.03% H2O2 diluted in 50 mmol/l Tris-HCl, pH 7.36), as chromogen and Harris hematoxylin solution (Sigma) as counterstaining. Sections were dehydrated, coverslipped, and observed under a Zeiss Axiovert 40 CFL optic microscope (Zeiss, Góttingen, Germany). Negative control slides without primary antibody were included to assess nonspecific staining.

Gelatin zymography

MMP-9, MMP-2, and MMP-9/LCN2 gelatinolytic activities were measured as previously described [31]. Briefly, protein extracts of 15 µg from each sample were run in duplicate in 10% SDS-PAGE containing 0.1% gelatin (Sigma). After the electrophoresis, gels were washed in 2.5% Triton X-100 (Sigma) for 45 min and subsequently incubated overnight at 37°C in enzyme development buffer (Invitrogen). After incubation, gels were fixed in 50% (v/v) methanol and 7% (v/v) acetic acid (Sigma) for 15 min and then stained for 1 h in GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA). Finally, the gels were rinsed in distilled water. MMP-9, MMP-2, and LCN2/MMP-9 complex were identified based on their molecular weight, and Quantity One software (Bio-Rad) was used for densitometric analysis of the zymographic activities.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences between the LN and OB groups were assessed by two-tailed unpaired Student's t test. Due to their non-normal distribution gene and protein expression levels were logarithmically transformed. The normal distribution of the other variables was adequate for the use of parametric tests. Pearson's correlation coefficients (r) were used to analyze the association between variables. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS, Chicago, IL, USA). A $p<0.05$ was considered statistically significant.

RESULTS

Gene and protein expression levels of LCN2 in visceral adipose tissue are elevated in human obesity

The biochemical and hormonal characteristics of the subjects included in the expression study are shown in Table 1. As expected, obese patients showed significantly higher BMI ($p<0.0001$) and BF ($p<0.0001$) compared to the lean volunteers. The high BF values were accompanied by increased leptin concentrations ($p<0.0001$) and reduced circulating concentrations of HDL cholesterol ($p<0.001$). Obese patients exhibited lower insulin sensitivity than lean individuals as evidenced by the increased HOMA ($p< 0.001$) and decreased QUICKI ($p=0.011$) indices. The circulating levels of the inflammatory markers CRP ($p< 0.0001$) and fibrinogen ($p=0.002$) were higher in the obese patients as compared to the lean volunteers.

Real-time PCR analysis indicated that LCN2 mRNA expression levels in VAT were significantly higher in obese subjects (5.3-fold change; $p<0.0001$), compared to lean controls (Fig. 1a). Moreover, a positive correlation was found between gene expression levels of LCN2 and BMI ($r=0.40$; $p=0.012$). LCN2 protein levels in VAT followed a similar trend, being significantly increased in obese patients ($p=0.017$; Fig. 1b). Furthermore, a positive correlation ($r= 0.50$; $p=0.010$) between gene and protein LCN2 expression levels in VAT was found.

The presence of LCN2 in sections of VAT was confirmed by immunohistological analysis (Fig. 1c).

Both adipocytes and cells of the stromovascular fraction were immunopositive for LCN2. As expected, CD68 staining in adipose tissue was localized mainly to macrophages. LCN2 labeling was detected in CD68-positive cells, but a marked staining was also observed in mature adipocytes.

Increased circulating concentrations of the LCN2/MMP-9 complex in human obesity

Baseline characteristics of subjects included in the study of circulating concentrations of LCN2 are summarized in Table 2. No significant differences were detected in circulating LCN2 between groups (Fig. 2a). In addition, no significant correlation of circulating LCN2 with either gene ($r=-0.19$; $p=0.430$) or protein ($r=-0.09$; $p=0.861$) expression levels in VAT was observed in the common samples of both studies (n=8 for the lean group and n=27 for obese volunteers). However, increased circulating concentrations of the LCN2/MMP-9 complex was detected in the obese group compared to lean subjects ($p=0.038$; Fig. 2b). LCN2/MMP-9 complex concentrations were positively correlated with BMI ($r=0.49$; $p=0.003$), leptin ($r=0.50$; $p=0.025$), and inflammatory markers CRP ($r= 0.74$; $p<0.001$) and fibrinogen ($r=0.61$; $p<0.001$), but these associations were lost after adjusting for BF.

Matrix metalloproteinases activity in adipose tissue

In order to assess the involvement of LCN2 in MMPs regulation, the activities of MMP-9, MMP-2, and the LCN2/MMP-9 complex were determined in adipose tissue homogenates using zymography. The activity of MMP-2 and the MMP-9/LCN2 complex were significantly increased in obese patients compared to lean subjects ($p< 0.01$), whereas no significant differences were detected for the activity of MMP-9 (Fig 3). The activity of the MMP-9/ LCN2 complex was significantly correlated ($p<0.05$) with mRNA expression of MMP-9 and LCN2 after BF adjustment (Table 3). The activities of MMP-2 and MMP-9 were significantly correlated with their respective gene expression levels ($p<0.01$). Moreover, gene expression levels of MMP-9 and MMP-2 were significantly correlated ($p<0.05$) with mRNA expression levels of OPN, a protein involved in matrix remodeling.

LCN2 expression levels are related to inflammatory markers in VAT

To address the relevance of inflammation, gene expression levels of MMP-2, MMP-9, OPN, and TNF-α were measured in VAT in the obese subjects compared to the group of lean volunteers. As macrophages are an important cell type producing pro-inflammatory cytokines, the macrophage-specific marker CD68 was used to evaluate the impact of resident macrophages in adipose tissue. Expression levels of CD68 (2.7-fold change; $p=0.014$), MMP-9 (10.6-fold change; $p<0.0001$), OPN (7.6-fold change; $p<0.001$), and TNF-α (3.2-fold change; $p=0.025$) were upregulated in obese subjects. A similar tendency was observed in MMP-2 mRNA expression, but the differences did not reach statistical significance (1.7-fold change; $p=0.257$; Fig. 4).

After adjusting for BF, a highly significant positive correlation between mRNA expression of LCN2 and all the pro-inflammatory genes studied (CD68, MMP-9, MMP-2, OPN, and TNF-α; $p<0.01$; Table 4) was found. Moreover, LCN2 protein levels were positively correlated ($p<0.05$) with mRNA expression of MMP-9, OPN, and TNF-α and also with the circulating inflammatory markers fibrinogen and CRP ($p<0.01$; Table 4).

DISCUSSION

Adipose tissue acts as an active autocrine, paracrine, and endocrine organ, secreting an increasing number of adipokines that participate in diverse metabolic processes [4, 32]. In this sense, LCN2 has been recently described as an adipokine involved in inflammation and insulin resistance. The main findings of this study are (1) that mRNA and protein expression levels of LCN2 are upregulated in VAT in human obesity, (2) that the circulating concentrations and activity of the LCN2/ MMP-9 complex are increased in obese patients without changes in LCN2 concentrations, and (3) that gene and protein expression levels of LCN2 in VAT are related to pro-inflammatory markers.

We detected an increased mRNA and protein expression of LCN2 in human VAT in obesity, which is in accordance with previous studies in animal models of obesity, where upregulated expression levels were detected in both adipose tissue and liver of db/db mice and in isolated adipocytes from obese Zucker rats [12, 14]. Although LCN2 is expressed in most cellular types, it has been suggested that adipocytes may be the main source of LCN2 expression in mice [13]. Furthermore, LCN2 expression is increased after conversion of preadipocytes to mature adipocytes [14]. However, another study showed higher mRNA expression levels of LCN2 in the nonfat cells of human omental adipose tissue [33]. In the present study, we have demonstrated the presence of LCN2 by immunohistochemistry in human visceral adipose tissue. LCN2 labeling was detected in CD68-positive macrophages as might be expected, but a marked staining in fully matured adipocytes was also shown.

Our study provides evidence that obese patients do not exhibit increased circulating concentrations of LCN2, thus, contrasting with previously published data in both human [12] and rodent models of obesity [13]. A similar controversy has arisen in relation to other studies with another member of the lipocalin family, retinol binding protein 4, which is also produced by adipose tissue with increased circulating levels in obesity and T2DM not being univocal [34, 35]. The increased expression of LCN2 in VAT and the lack of changes in its circulating concentrations suggest an autocrine/paracrine function of LCN2 in agreement with a very recently reported work [14]. A large body of evidence shows that visceral obesity is associated with a higher risk of obesity-related comorbidities whereas subcutaneous fat deposition has been associated with a decreased risk of cardiovascular diseases [36, 37]. In this sense, visceral adipocytes are more metabolically active and display huge differences in gene expression levels than subcutaneous adipocytes. In light of the divergent pathological consequences of differences in adipose tissue distribution [38, 39], it would be of interest to analyze the molecular characteristics underlying the diverse secretory profile of VAT and subcutaneous adipose tissue together with their impact on LCN2 expression. Two cell-surface LCN2 receptors have been described: megalin/gp330 [40] and 24p3R [9], with the latter being more specific for LCN2 binding. However, gene expression of 24p3R has not been detected in adipose tissue from mice or in 3T3-L1 cells [13] and signaling pathways triggered by these receptors remain unknown. The study of these and other possible receptors for LCN2 may help to better understand the role of LCN2 in adipose tissue.

On the other hand, LCN2 has been described to exert a protective effect on MMP-9, preventing the latter from degradation in cell cultures [24]. In this regard, we found increased circulating concentrations of the LCN2/MMP-9 complex in obese patients. It may explain the lack of differences in the circulating concentrations of LCN2 taking into account that the association of LCN2 and MMP-9 may prevent its release into circulation. Furthermore, given the enhanced activity of the LCN2/MMP-9 complex detected in VAT in the obese group as well as the correlation with BMI, LCN2 may be exerting an indirect active role in obesity progression through MMP9 protection. Development of obesity is associated with modifications in adipose tissue involving adipogenesis, angiogenesis, and proteolysis of the extracellular matrix. Moreover, the basement membrane of adipocytes has to be degraded in order to allow the hypertrophic development observed in obesity. The MMP system contributes to adipose tissue remodeling with MMP-2 and MMP-9 expression shown in human adipose tissue [23, 41]. Although we showed higher MMP-

2 activity in the VAT from the obese group, no changes in MMP-9 activity were detected. On the contrary, upregulation of MMP-9 mRNA was detected in obese patients, but no differences were evident in MMP-2 gene expression. It has been described that during adipose differentiation, MMP-2 mRNA expression increases in a constant manner, whereas MMP-9 expression shows a different profile, with a strong downregulation at the end of the differentiation period [23]. This different behavior may underlie the inverse profile between activity and gene expression levels of both MMPs observed in our study. OPN, an extracellular matrix protein, plays a significant role in cell adhesion, migration, and extracellular matrix degradation [42]. It has been shown that OPN provides a paracrine signal that elevates vascular pro-MMP-9 activity [43] and induces pro-MMP-2 production and activation [44]. In this regard, a significant correlation between gene expression levels of OPN, and MMP-9 and MMP-2 mRNA expression levels were also observed.

Chronic low-grade inflammation is known to be a mediator in the development of obesity-related diseases. In agreement with previous results, we have shown an upregulation of mRNA expression levels of several key pro-inflammatory genes such as TNF-α, MMP-2, MMP-9, and OPN, in obesity [22, 23, 45, 46] establishing a significant association of these markers with gene expression levels of LCN2. In line with this observation, LCN2 protein levels were positively correlated with CRP and fibrinogen, well-established markers of chronic inflammation. Wang et al. [12] observed an association between serum LCN2 and CRP (both increased in obese subjects) with rosiglitazone, a drug with anti-inflammatory activities, decreasing lipocalin concentrations [12]. It has been described that LCN2 is induced by TNF-α [13] a relevant factor in inflammatory processes. In line with this functional relation, we have established a significant positive association between LCN2 and TNF-α mRNA in VAT. These data suggest the implication of LCN2 in lowgrade inflammation associated to obesity in humans, in particular at the autocrine/paracrine level in VAT. In vitro studies have shown that LCN2 induces mRNA levels of PPARγ and adiponectin, attenuates the effect of TNF-α on inflammation, and decreases cytokine expression in macrophages after LPS stimulation antagonizing the effects of inflammatory molecules [14]. In this regard, it has been also described that the LCN2/siderophore/iron complex protects the kidney and mitigates azotemia in a mouse model of severe renal failure with ischemiareperfusion injury sequestering neutrophil chemoattractants and abating neutrophil-dependent inflammatory responses [47]. Taken together, these data indicate that LCN2 may act as a negative regulator of the effect of inflammatory molecules.

In summary, we show for the first time that LCN2 mRNA and protein expression in human VAT is increased in obese patients. Moreover, the correlation between both LCN2 gene and protein expression with different proinflammatory markers highlights an involvement in the low-grade chronic inflammation accompanying obesity likely acting as an anti-inflammatory molecule. The unchanged circulating concentrations of LCN2 in obesity reported herein point to a more likely autocrine/paracrine role of LCN2 in adipose tissue or a modulation of protease activity by protecting MMP-9 from degradation. Further studies to better understand the implication of this adipokine in obesity are warranted.

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Table 1 Anthropometric and biochemical characteristics of subjects included in the gene and protein expression study

	Lean	Obese	p Value
n (female, male)	8 (5, 3)	39 (19, 20)	
Age (years)	40±4	41±2	ns
BMI (kg/m^2)	21.4±1.0	43.8±1.1	<0.0001
Body fat (%)	26.1±2.3	48.1±1.3	<0.0001
Fasting glucose (mmol/l)	4.9±0.2	5.8±0.5	ns
2h OGTT glucose (mmol/l)	5.6±0.8	8.0±0.9	ns
Fasting insulin (pmol/l)	54.3±11.6	150.6±18.1	<0.001
2h OGTT insulin (pmol/l)	61.5±13.8	692.2±86.9	<0.0001
HOMA	1.7±0.3	6.0±1.1	<0.001
QUICKI	0.363±0.014	0.314±0.008	0.011
Triglycerides (mmol/l)	0.7±0.1	1.9±0.4	ns
Cholesterol (mmol/l)	4.8±0.3	5.2±0.2	ns
LDL cholesterol (mmol/l)	3.1±0.2	3.4±0.2	ns
HDL cholesterol (mmol/l)	1.6±0.1	1.1±0.1	0.001
Leptin (ng/ml)	7.8±1.2	40.0±6.2	<0.0001
Uric acid (mg/dl)	4.0±0.4	6.3±0.4	0.011
C-reactive protein (mg/l)	1.7±0.3	6.3±1.0	<0.0001
Fibrinogen (mg/dl)	219±39	341±14	0.002
vWF (%)	104±14	123±11	ns
Homocysteine (µmol/l)	7.0±0.9	8.2±0.5	ns
ALT (UI/l)	11±3	35±4	<0.0001
AST (UI/l)	13±3	18±1	ns
Alkaline phosphatase (UI/l)	103±10	112±8	ns
γ-GT (UI/l)	10±2	29±7	ns

Data are mean ± SEM. Differences between the LN and OB groups were assessed by two-tailed unpaired Student's *t* test

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* body mass index, *HOMA* homeostatic model assessment, *OGTT* oral glucose tolerance test, *QUICKI* quantitative insulin-sensitivity check index, *vWF* von Willebrand factor, *γ-GT* γ-glutamyltransferase

Table 2 Anthropometric and biochemical characteristics of subjects included in the circulating levels study

	Lean	Obese	p Value
n (female/male)	13 (7, 6)	27 (14, 13)	
Age (years)	31±3	38±2	ns
BMI (kg/m^2)	21.6±1.0	44.8±1.6	<0.0001
Body fat (%)	21.4±1.4	49.2±1.7	<0.0001
Fasting glucose (mmol/l)	4.7±0.3	5.7±0.7	ns
2h OGTT glucose (mmol/l)	5.6±0.8	9.2±1.1	ns
Fasting insulin (pmol/l)	45.6±9.4	153.5±21.0	0.001
2h OGTT insulin (pmol/l)	61.5±14.1	738.4±76.4	<0.0001
HOMA	1.4±0.2	7.0±1.3	<0.01
QUICKI	0.373±0.011	0.309±0.007	<0.0001
Triglycerides (mmol/l)	0.8±0.1	2.1±0.6	ns
Cholesterol (mmol/l)	4.3±0.2	5.3±0.3	0.024
LDL cholesterol (mmol/l)	2.4±0.1	3.4±0.2	<0.001
HDL cholesterol (mmol/l)	1.6±0.2	1.1±0.5	<0.001
Leptin (ng/ml)	6.9±1.6	58.4±8.8	<0.001
Uric acid (mg/dl)	4.2±0.1	5.8±0.5	0.020
C-reactive protein (mg/l)	0.9±0.4	6.9±1.1	<0.001
Fibrinogen (mg/dl)	181±13	323±17	<0.0001
vWF (%)	52±9	117±13	0.002
Homocysteine (µmol/l)	6.9±0.6	8.1±0.6	ns
ALT (UI/l)	6±1	35±5	<0.001
AST (UI/l)	13±1	17±2	ns
Alkaline phosphatase (UI/l)	83±8	109±11	ns
γ-GT (UI/l)	11±1	35±10	ns

Data are mean ± SEM. Differences between the LN and OB groups were assessed by two-tailed unpaired Student's *t* test

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *BMI* body mass index, *HOMA* homeostatic model assessment, *OGTT* oral glucose tolerance test, *QUICKI* quantitative insulin-sensitivity check index, *vWF* von Willebrand factor, *γ-GT* γ-glutamyltransferase

Table 3 Univariate analysis of the correlation between MMPs activities in VAT and other variables after BF adjustment

BF adjustment	MMP-9 activity		MMP-2 activity		MMP-9/LCN2 activity	
	r	*p* Value	*r*	*p* Value	*r*	*p* Value
MMP-9 activity	–	–	0.62	0.002	0.51	0.016
MMP-2 activity	0.62	0.002	–	–	0.97	<0.001
MMP-9/LCN2 activity	0.51	0.016	0.97	<0.001	–	–
mRNA *MMP-9*	0.56	0.007	0.44	0.040	0.45	0.037
mRNA *MMP-2*	0.45	0.038	0.55	0.008	0.55	0.008
mRNA *LCN2*	0.61	0.002	0.53	0.011	0.53	0.012
BMI	0.46	0.030	0.48	0.024	0.55	0.007

Table 4 Univariate analysis of the correlation between *LCN2* mRNA and protein expression levels in VAT and inflammatory markers after BF adjustment

BF adjustment	mRNA LCN2		Protein LCN2	
	r	*p* Value	*r*	*p* Value
mRNA *LCN2*	–	–	0.52	0.013
Protein LCN2	0.52	0.013	–	–
mRNA *TNF-α*	0.59	<0.001	0.49	0.024
mRNA *MMP-9*	0.49	0.002	0.49	0.019
mRNA *MMP-2*	0.48	0.002	0.40	0.062
mRNA *OPN*	0.40	0.018	0.44	0.043
mRNA *CD68*	0.58	<0.001	0.31	0.161
Fibrinogen	0.29	0.150	0.74	0.003
C-reactive protein	0.23	0.264	0.66	0.008

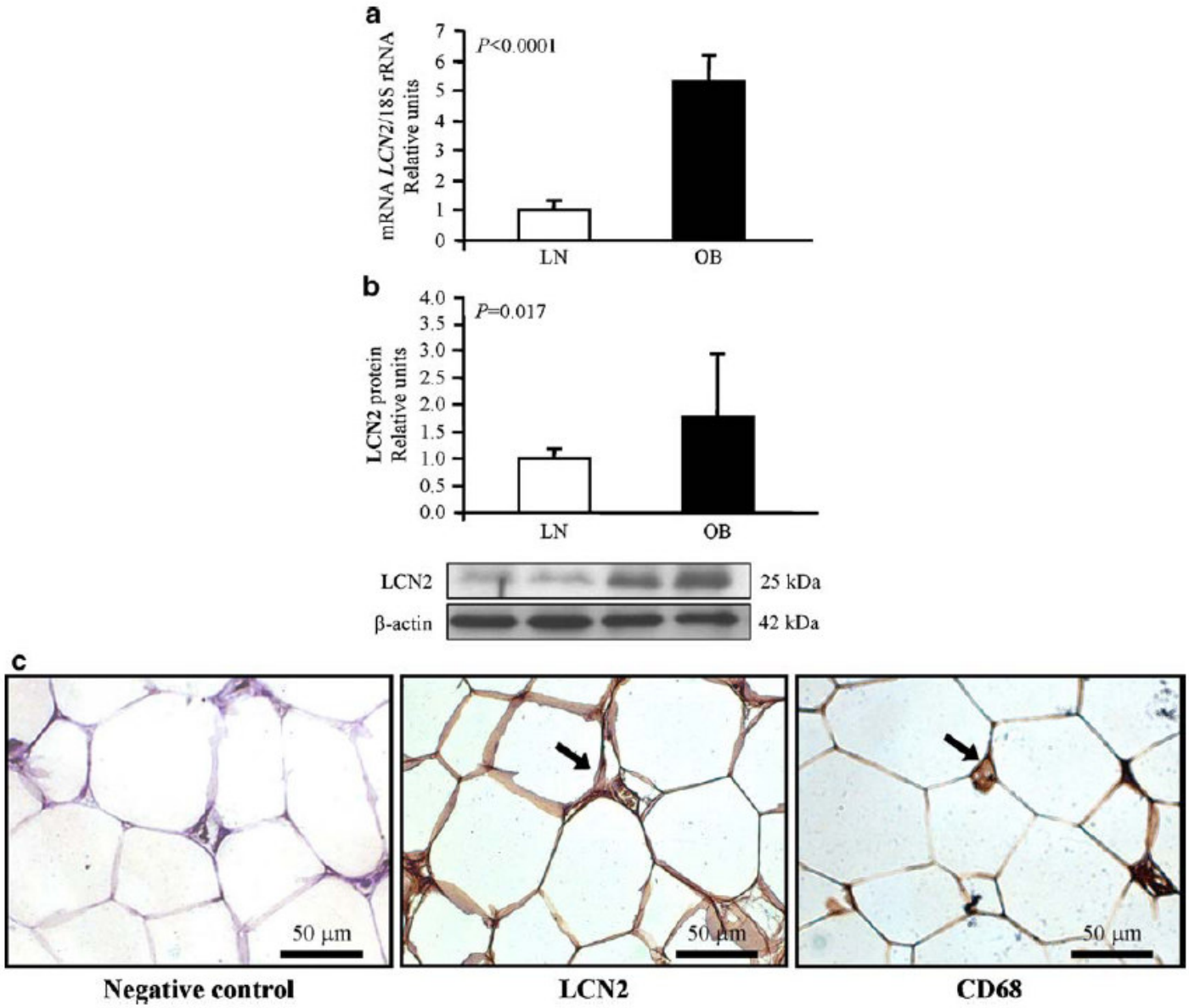

Fig. 1 Gene (**a**) and protein (**b**) expression of LCN2 of lean (LN) and obese (OB) volunteers and immunohistochemistry (**c**) of LCN2 and CD68 in visceral adipose tissue (VAT). **a** Real-time PCR analysis of *LCN2*. Bars represent the mean ± SEM of the ratio between gene expression to *18S* rRNA. The expression level in lean subjects was assumed to be 1. Differences between the LN and OB groups were assessed by two-tailed unpaired Student's *t* test (LN: $n=8$; OB: $n=39$). **b** Western-blot analysis of LCN2 levels. Bars represent the mean ± SEM of the ratio between LCN2 to β-actin. The expression of LCN2 in LN subjects was assumed to be 1. Representative blots in duplicate are shown at the bottom of the figure. Differences between the LN and OB groups were assessed by two-tailed unpaired Student's *t* test (LN: $n=5$; OB: $n=20$). **c** Immunohistochemistry of LCN2 and CD68 in VAT. Strong positivity (brown staining) was observed for LCN2 in both fully mature adipocytes and cells of the stromovascular fraction. No immunoreactivity was found without the primary antibody (negative control). Images are representative of immunostaining in VAT from six obese subjects. (*Scale bar*=50 μm)

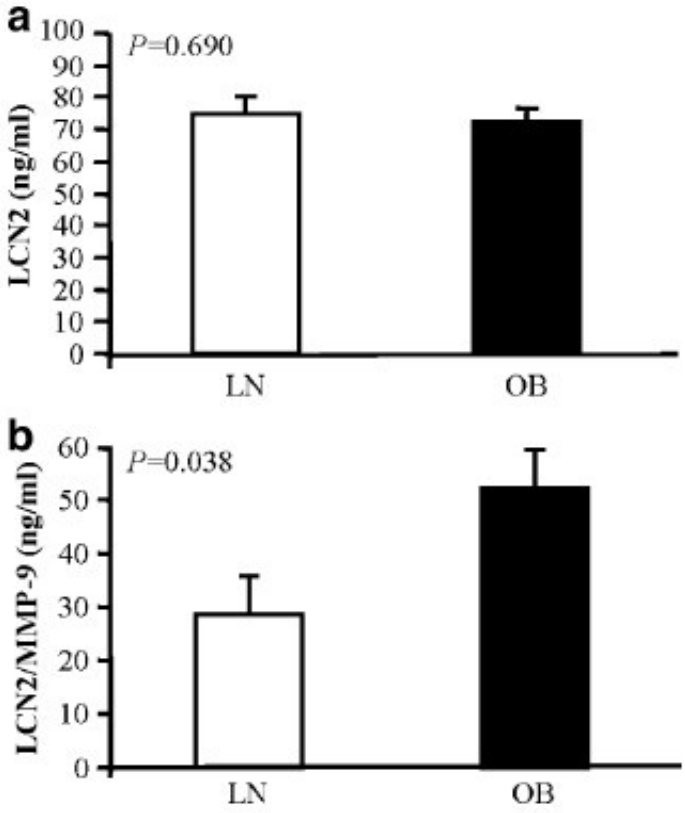

Fig. 2 Circulating concentrations of LCN2 (**a**) and the LCN2/MMP-9 complex (**b**) in lean (LN) and obese (OB) volunteers. Differences between the LN and OB groups were assessed by two-tailed unpaired Student's *t* test (LN: $n=13$; OB: $n=27$)

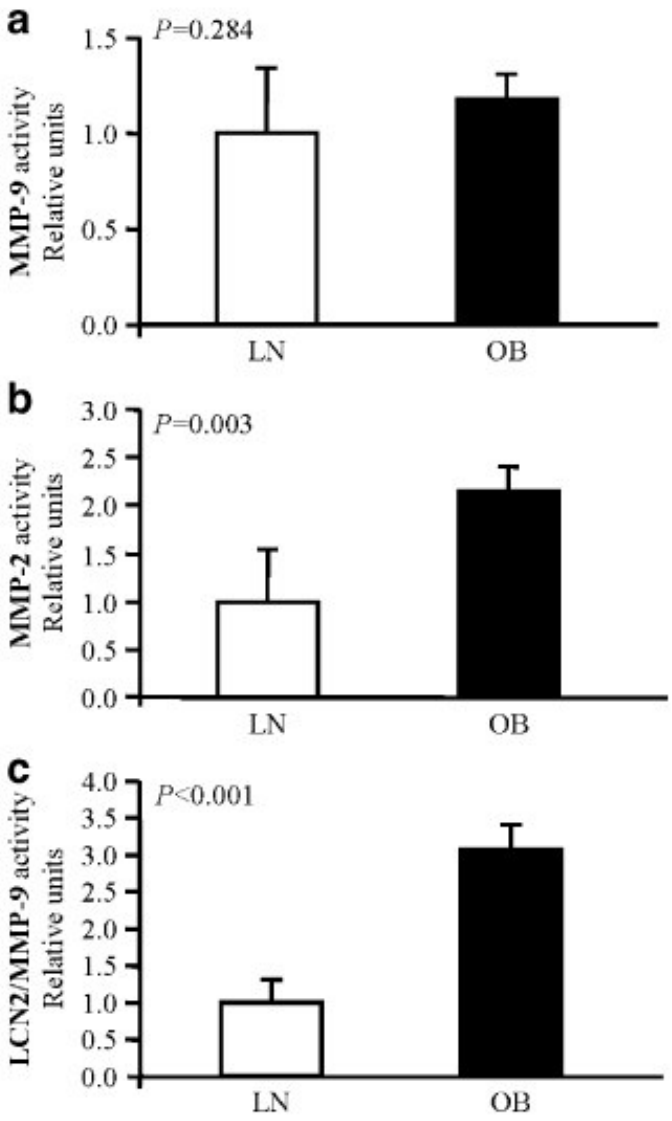

Fig. 3 Zymography analysis of MMP-9 (**a**), MMP-2 (**b**), and MMP-9/LCN2 complex (**c**) activity levels in VAT obtained of lean (LN) and obese (OB) volunteers. The activity in LN subjects was assumed to be 1. Differences between the LN and OB groups were assessed by two-tailed unpaired Student's *t* test (LN: $n=5$; OB: $n=20$)