

Plasma Osteopontin Levels and Expression in Adipose Tissue Are Increased in Obesity

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Context: Obesity acts as a cardiovascular risk factor by mechanisms that are not fully understood. Osteopontin (OPN) is a proinflammatory mediator involved in tissue remodeling that plays a role in atherosclerosis and diabetes.

Objective: The aim of the present study was to compare the circulating concentrations of OPN and its mRNA expression in omental adipose tissue of lean, overweight, and obese individuals and to analyze the effect of weight loss.

Subjects and Methods: Plasma concentrations of OPN were measured in 77 volunteers. *OPN* mRNA expression in omental adipose tissue obtained from 12 women was quantified by real-time PCR. In addition, the concentrations of OPN in 12 obese men were measured before and after weight loss following a dietetic program.

Setting: The study was conducted at a University Hospital.

Results: Obese and overweight patients exhibited significantly increased circulating OPN concentrations as compared with lean sub-

jects (obese 72.6 ± 28.5 , overweight 68.2 ± 20.8 , lean 42.7 ± 27.9 ng/ml; $P < 0.001$). A significant positive correlation was found between OPN levels and body fat ($r = 0.45$; $P < 0.0001$). Obese individuals showed significantly increased ($P < 0.05$) mRNA expression of *OPN* in omental adipose tissue as compared with lean volunteers, which was further increased in obese diabetic patients. Diet-induced weight loss significantly decreased OPN concentrations from 64.7 ± 22.1 to 36.6 ± 20.1 ng/ml ($P = 0.006$).

Conclusions: These findings represent the first observation that plasma OPN and mRNA expression of *OPN* in omental adipose tissue are increased in overweight/obese patients with the latter being further elevated in obesity-associated diabetes. Moreover, weight loss reduces OPN concentrations, which may contribute to the beneficial effects accompanying weight reduction. Measurement of OPN might be useful for evaluating the outcomes of various clinical interventions for obesity-related cardiovascular diseases. (*J Clin Endocrinol Metab* 92: 3719–3727, 2007)

OBESITY IS ASSOCIATED with alterations in myocardial and vascular structure as well as physiology, which are accompanied by an adverse risk factor profile leading to the development of cardiovascular disease (1–3). This circumstance favors the clustering of metabolic and cardiovascular alterations leading to an increase in morbidity and mortality in relation to excess adiposity (4–6).

Osteopontin (OPN), also known as early T lymphocyte activation (Eta-1), secreted phosphoprotein-1 (SPP1), and bone sialoprotein-1 among others, is a phosphoprotein expressed by a wide variety of cell types, such as osteoclasts, lymphocytes, endothelial cells, macrophages, epithelial cells, hepatocytes, and vascular smooth muscle cells. OPN signals through integrin receptors and isoforms of CD44 (also

known as extracellular matrix receptor type III), whereby it is involved in several pathophysiological processes including bone remodeling, immunity, inflammation, neoplastic transformation, progression of metastases, promotion of cell survival, and wound healing (7, 8).

In addition, OPN has been shown to exert an impact on the cardiovascular sphere, playing a role in atherosclerosis (9–12), left ventricular hypertrophy (13), and cardiac fibrosis (14), processes frequently associated with obesity (15–17). Furthermore, *OPN* mRNA expression is up-regulated in the heart of infarcted obese mice (18). OPN has also been involved in diabetic vascular disease (9, 19) as well as in the development of nonalcoholic steatohepatitis (20). Expression of OPN in adipose-derived stem cells, but not in adipocytes, has been previously reported (21). However, to date, neither circulating concentrations of OPN in human obesity nor *OPN* expression in adipose tissue has been investigated.

The present study explores the actual plasma levels of OPN as well as the mRNA expression of *OPN* in omental adipose tissue in human obesity. We hypothesized that plasma OPN concentrations will be increased in accordance with the degree of adiposity. To validate this assumption further, we assessed the effect of weight loss on plasma OPN levels.

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Abbreviations: ALT, Alanine aminotransferase; AST, aspartate aminotransferase; BMD, bone mineral density; BMI, body mass index; CRP, C-reactive protein; γ -GT, γ -glutamyltransferase; HDL, high-density lipoprotein; IGT, impaired glucose tolerance; LDL, low-density lipoprotein; MMP, matrix metalloproteinase; NG, normoglycemia; OGTT, oral glucose tolerance test; OPN, osteopontin; QUICKI, quantitative insulin sensitivity check index; TBST, Tris-buffered saline Tween 20; T2DM, type 2 diabetes mellitus; vWF, von Willebrand factor.

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Patients and Methods

Patient selection and study design

To analyze the effect of obesity on OPN concentrations, 77 Caucasian subjects (26 lean, 14 overweight, and 37 obese) were recruited from healthy volunteers and patients attending the Endocrinology and Surgery Departments at the University Clinic of Navarra. Subjects were classified according to body mass index (BMI) (lean, <25; overweight, 25–29.9; and obese, ≥ 30 kg/m²). Patients underwent a clinical assessment including medical history, physical examination, body composition analysis, and comorbidity evaluation as well as nutritional interviews performed by a multidisciplinary consultation team. All subjects were nonsmokers. Patients with signs of infection were excluded. Obese patients were not receiving statins or antidiabetic medication. Normoglycemia (NG), impaired glucose tolerance (IGT), and type 2 diabetes mellitus (T2DM) are defined following the criteria of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (22) based on both fasting plasma glucose concentrations and plasma glucose 2 h after an oral glucose tolerance test (OGTT).

In addition, mRNA expression of OPN in omental adipose tissue was assessed in a separate group of female obese patients after a Roux-en-Y gastric bypass (n = 8) and in a group of lean patients undergoing Nissen fundoplication (n = 4) for the treatment of hiatus hernia.

Furthermore, the effect of weight loss on plasma OPN concentrations in a different group of 12 obese male patients was analyzed. Weight loss was achieved by prescription of a diet providing a daily energy deficit of 500–1000 kcal/d as calculated from the determination of the resting energy expenditure through indirect calorimetry (Vmax29; SensorMedics Corp., Yorba Linda, CA) and multiplication by the physical activity level factor to obtain the individual's total energy expenditure. This hypocaloric regime allows a safe and steady weight loss of 0.5–1.0 kg/wk when strictly followed and supplied 30, 54, and 16% of energy requirements in the form of fat, carbohydrates, and protein, respectively.

The experimental design was approved, from an ethical and scientific standpoint, by the Hospital's Ethical Committee responsible for research, and volunteers gave their informed consent to participate in all the studies.

Anthropometric measurements

Body weight was measured with a digital scale to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm with a Holtain stadiometer (Holtain Ltd., Crymych, UK). BMI was calculated as weight in kilograms divided by the square of height in meters. Body fat was estimated by air-displacement plethysmography (Bod-Pod; Life Measurements, Concord, CA). Data for estimation of body fat by this plethysmographic method has been reported to agree closely with the traditional gold standard hydrodensitometry (underwater weighing). Furthermore, the Bod-Pod has been shown to predict fat mass and fat-free mass more accurately than dual-energy x-ray absorptiometry and bioelectrical impedance (23–25). Blood pressure was measured after a 5-min rest in the semi-sitting position with a sphygmomanometer. Blood pressure was determined at least three times at the right upper arm, and the mean was used in the analyses.

Analytical procedures

Blood samples were collected after an overnight fast in the morning to avoid potential confounding influences due to hormonal rhythmicity. Plasma glucose was analyzed by an automated analyzer (Roche/Hitachi Modular P800) as previously described (26, 27). Insulin was measured by means of an enzyme-amplified chemiluminescence assay (IMMULITE; Diagnostic Products Corp., Los Angeles, CA). An indirect measure of insulin sensitivity was calculated from the fasting plasma glucose and insulin concentrations by using the quantitative insulin sensitivity check index (QUICKI) (28, 29). Total cholesterol and triglyceride concentrations were determined by enzymatic spectrophotometric methods (Roche, Basel, Switzerland). High-density lipoprotein (HDL)-cholesterol was quantified by a colorimetric method in a Beckman Synchron CX analyzer (Beckman Instruments, Ltd., Bucks, UK). Low-density lipoprotein (LDL)-cholesterol was calculated by the Friedewald formula.

Uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, γ -glutamyltransferase (γ -GT), and cre-

atinine were measured by enzymatic tests (Roche) in an automated analyzer (Roche/Hitachi Modular P800). Fibrinogen concentrations were determined according to the method of Clauss using a commercially available kit (Hemoliance; Instrumentation Laboratory, Barcelona, Spain). Measurement of von Willebrand factor (vWF) antigen was performed by a micro-latex immunoassay (Diagnostica Stago, Inc., Parsippany, NJ). A standard curve was prepared with a universal reference (NISBC 91/666), and the results were expressed as percentage of the standard. Intra- and interassay coefficients of variation were 4.0 and 8.0%, respectively. High-sensitivity C-reactive protein (CRP) was measured using the Tina-quant CRP (Latex) ultrasensitive assay (Roche). Leptin was quantified by a double-antibody RIA method (Linco Research, Inc., St. Charles, MO); intra- and interassay coefficients of variation were 5.0 and 4.5%, respectively. Adiponectin was measured by ELISA (BioVendor, Brno, Czech Republic); intra- and interassay coefficients of variation were 6.7 and 7.8%, respectively. TNF- α was quantified by a high-sensitivity ELISA (R&D Systems, Abingdon, UK); intra- and interassay coefficients of variation were 6.7 and 13.4%, respectively. OPN was determined by ELISA (R&D Systems) with intra- and interassay coefficients of variation being 3.2 and 5.9%, respectively.

Bone mineral density (BMD)

In a subset of obese patients (n = 20), BMD was measured by dual x-ray absorptiometry using a Hologic QDR4500-W densitometer (Hologic, Inc., Bedford, MA). The area of BMD in grams per square centimeter was measured at the lumbar spine (L2–L4) as well as at the femoral neck, Ward's triangle, trochanter, and total hip. The manufacturer's software calculated bone mineral content and areal BMD for each vertebra. The average BMD of L2–L4 were determined by averaging the values for L2, L3, and L4. The time required for the procedure was approximately 5 min, and the radiation exposure was negligible. Osteoporosis and osteopenia were defined, according to the World Health Organization criteria at any measured site relative to age- and sex-related reference values (30).

OPN and CD68 immunohistochemistry in adipose tissue

Immunohistochemistry was carried out using the osteopontin (human) immunohistochemistry staining kit (Bachem AG, Bubendorf, Switzerland) following the manufacturer's instructions or following a previously described protocol for CD68 (31). Briefly, sections (6 μ m) of formalin-fixed, paraffin-embedded omental adipose tissue from an obese subject were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol and tap water. Subsequently, sections were immersed in 10 mmol/liter citrate buffer (pH 6.0) and heated using a microwave oven at 800 W for 15 min to enhance antigen retrieval. After cooling, sections were treated with 3% H₂O₂ in water for 20 min for quenching of endogenous peroxidase activity. Then, slides were blocked during 15 min with 100 μ l normal goat serum blocking solution (OPN) or murine serum (Sigma Chemical Co., St. Louis, MO) (CD68) to prevent nonspecific adsorption. Sections were incubated overnight at 4 C with 100 μ l rabbit antihuman OPN antibody raised against a peptide corresponding to residues 170–187 of human OPN (Bachem) or monoclonal mouse antihuman CD68 antibody (M0876; DakoCytomation, Glostrup, Denmark) at a concentration of 10 μ g/ml. Sections were washed three times (2 min each) with Tris-buffered saline Tween 20 (TBST) and then incubated for 45 min at RT with 100 μ l biotinylated goat antirabbit secondary antibody for OPN (Bachem) or sheep antimouse polyclonal IgG for CD68 (NXA931; Amersham, Buckinghamshire, UK) diluted 1:100 in TBST. After washing in TBST, sections were incubated with 100 μ l of streptavidin-horseradish peroxidase conjugate for 30 min. Sections were washed three times with TBST and incubated 3 min with 100 μ l substrate-chromogen mixture (3,3'-diaminobenzidine/H₂O₂ solution) and counterstained with 100 μ l Mayer's hematoxylin for 2 min. Sections were washed in tap water, immersed in PBS (pH 7.4), rinsed in distilled water, coverslipped, and observed under a Zeiss Axiovert 40 CFL optic microscope (Zeiss, Göttingen, Germany). Negative control slides without primary antibody were included to assess nonspecific staining.

OPN and CD68 mRNA expression quantification by real-time PCR

Omental adipose tissue was obtained from 12 female patients undergoing either Roux-en-Y gastric bypass ($n = 8$) or Nissen fundoplication ($n = 4$). The samples were immediately frozen in liquid nitrogen and stored at -80°C . RNA isolation was performed as previously described (32). Transcript levels for *OPN* (*SPP1*) and *CD68* genes were quantified by real-time PCR (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA). Primers and probes (Sigma) were designed using the software Primer Express 1.0 (Applied Biosystems). Primers used to amplify the cDNA of *SPP1* (GenBank NM_001040060) were 5'-ACAGCCACAAGCAGTCCAGATTA-3' and 5'-TCCTGACTATCAATCACATCGGAAT-3', and the probe was 5'-FAM-AGCG-GAAAGCCAATGATGAGAGCAATG-TAMRA-3' and for *CD68* (GenBank NM_001251) were 5'-CACGCAGCACAGTGGACATT-3' and 5'-CGAGTTGCTGCAACTGAAGCT-3', and the probe was 5'-FAM-TCGGCTCAGAATGCATCCCTTCGA-TAMRA-3'. The cDNA was amplified at the following conditions: 95°C for 10 min, followed by 45 cycles of 15 sec 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 nmol/liter and 200 nmol/liter, respectively. All results were normalized to the levels of 18S rRNA (Applied Biosystems), and relative quantification was calculated using the $\Delta\Delta\text{Ct}$ formula (33). Relative mRNA expression was expressed as fold expression over the calibrator sample (average of gene expression corresponding to the lean group). All samples were run in triplicate and the average values were calculated.

Statistical analysis

Data are presented as mean \pm SD. Differences between groups were analyzed by ANOVA followed by Fisher's least significant difference tests for computing the differences between lean, overweight, and obese groups, by Kruskal-Wallis followed by *U* Mann-Whitney's tests for

analyzing the differences between groups in the expression study, and by two-tailed paired *t* tests for comparison of the weight loss effect. CRP and leptin concentrations were logarithmically transformed in the first study, because of their nonnormal distribution. The distribution of other variables was adequate for the application of parametric tests. Correlations between two variables were computed by Pearson's correlation coefficients (*r*) or Spearman's rank correlation coefficients (ρ) as appropriate. The calculations were performed using the SPSS version 13.0.1 (SPSS, Chicago, IL). A *P* value lower than 0.05 was considered statistically significant.

Results

Patient characteristics and metabolic profile

No statistically significant differences for gender distribution were found between the groups (Table 1). As expected, body fat was significantly higher ($P < 0.0001$) in the overweight and obese subjects, who also showed lower insulin sensitivity than lean individuals as evidenced by the lower QUICKI ($P < 0.0001$). However, increased concentrations of glucose ($P = 0.003$) and insulin ($P < 0.001$) were observed only in the obese group. Circulating concentrations of total cholesterol ($P < 0.0001$) and LDL-cholesterol ($P < 0.0001$) were significantly increased in the overweight and obese individuals, whereas HDL-cholesterol was reduced ($P < 0.0001$). The inflammatory markers CRP and TNF- α were significantly increased ($P < 0.0001$) in both overweight and obese patients. Fibrinogen, homocysteine, and vWF concentrations were higher in obese individuals ($P \leq 0.001$), together with an impaired hepatic function as evidenced by increased levels of transaminases, alkaline phosphatase, and

TABLE 1. Characteristics of lean, overweight, and obese subjects

	Lean	Overweight	Obese	<i>P</i>
n	26	14	37	
Sex (male/female)	11/15	7/7	19/18	0.769
Age (yr)	32.8 \pm 10.8	37.1 \pm 14.1	40.8 \pm 10.8 ^a	0.028
BMI (kg/m ²)	20.4 \pm 2.3	26.8 \pm 1.6 ^a	41.0 \pm 5.4 ^{a,b}	<0.0001
Body fat (%)	19.3 \pm 5.8	35.4 \pm 6.6 ^a	46.3 \pm 7.1 ^{a,b}	<0.0001
SBP (mm Hg)	106 \pm 13	106 \pm 11	129 \pm 16 ^{a,b}	<0.0001
DBP (mm Hg)	68 \pm 9	66 \pm 6	81 \pm 11 ^{a,b}	<0.0001
Glucose (mmol/liter)	4.6 \pm 0.6	5.2 \pm 0.6	5.8 \pm 1.8 ^a	0.003
Insulin ($\mu\text{U/ml}$)	5.8 \pm 3.1	10.3 \pm 4.0	19.2 \pm 11.5 ^{a,b}	<0.0001
QUICKI	0.385 \pm 0.038	0.340 \pm 0.021 ^a	0.314 \pm 0.029 ^{a,b}	<0.0001
Triglycerides (mmol/liter)	0.7 \pm 0.3	1.3 \pm 0.9	1.9 \pm 2.2 ^a	0.019
Cholesterol (mmol/liter)	4.2 \pm 0.9	5.4 \pm 1.2 ^a	5.3 \pm 1.3 ^a	<0.001
LDL-cholesterol (mmol/liter)	2.3 \pm 0.7	3.2 \pm 1.0 ^a	3.3 \pm 1.0 ^a	<0.001
HDL-cholesterol (mmol/liter)	1.6 \pm 0.3	1.4 \pm 0.4 ^a	1.1 \pm 0.2 ^{a,b}	<0.0001
Uric acid (mg/dl)	4.1 \pm 0.8	4.9 \pm 1.2	6.6 \pm 1.4 ^{a,b}	<0.0001
Fibrinogen (mg/dl)	179 \pm 44	335 \pm 77 ^a	328 \pm 72 ^a	<0.0001
vWF (%)	81 \pm 29	96 \pm 31	124 \pm 39 ^a	<0.001
Homocysteine ($\mu\text{mol/liter}$)	6.2 \pm 1.4	8.0 \pm 1.03	8.9 \pm 3.4 ^a	0.001
CRP (mg/liter)	0.7 (0.4–1.0)	3.5 (0.4–5.9) ^a	7.4 (2.4–11.8) ^{a,b}	<0.0001
TNF- α (pg/ml)	1.51 \pm 0.42	2.12 \pm 0.92 ^a	2.16 \pm 1.10 ^a	0.021
ALT (IU/liter)	8 \pm 6	19 \pm 24 ^a	31 \pm 19 ^{a,b}	<0.0001
AST (IU/liter)	11 \pm 6	16 \pm 10	18 \pm 10 ^a	0.021
Alkaline phosphatase (IU/liter)	76 \pm 25	82 \pm 25	96 \pm 31 ^a	0.014
γ -GT (IU/liter)	11 \pm 3	15 \pm 10	31 \pm 23 ^{a,b}	<0.0001
Creatinine (mg/dl)	0.81 \pm 0.09	0.86 \pm 0.16	0.84 \pm 0.13	0.396
Adiponectin ($\mu\text{g/ml}$)	13.8 \pm 7.0	9.1 \pm 4.8 ^a	8.9 \pm 4.0 ^a	0.002
Leptin (ng/ml)	6.5 (4.3–9.6)	22.0 (9.4–29.7) ^a	47.3 (31.2–73.2) ^{a,b}	<0.0001

Data are presented as mean \pm SD or median (interquartile range). Differences between groups were analyzed by ANOVA followed by least significant difference tests. Differences in gender distribution were analyzed by χ^2 analysis. CRP and leptin concentrations were logarithmically transformed for statistical analysis. To convert glucose to mg/dl, divide by 0.05551; to convert values for triglycerides to mg/dl, divide by 0.01129; to convert values for cholesterol to mg/dl, divide by 0.02586. DBP, Diastolic blood pressure; SBP, systolic blood pressure.

^a $P < 0.05$ vs. lean.

^b $P < 0.05$ vs. overweight.

γ -GT. As expected, the increased body fat values of overweight and obese patients were accompanied by hypoadiponectinemia ($P = 0.002$) and hyperleptinemia ($P < 0.0001$).

Obesity and OPN concentrations

Circulating concentrations of OPN were significantly increased in obese and overweight patients (obese 72.6 ± 28.5 , overweight 68.2 ± 20.8 , lean 42.7 ± 27.9 ng/ml; $P < 0.001$) compared with lean subjects (Fig. 1). No differences between overweight and obese patients were observed ($P = 0.611$), probably due to the high body fat percentage displayed despite exhibiting a BMI within the range of 25–30 kg/m² by the overweight subjects (Fig. 2). OPN levels were slightly higher in obese individuals with IGT or T2DM ($n = 18$; 76.2 ± 31.6 ng/ml) compared with obese NG patients ($n = 19$; 69.1 ± 25.7 ng/ml), but the differences fell out of statistical significance ($P = 0.458$). No significant differences between male (60.2 ± 30.8 ng/ml) and female patients (63.1 ± 29.7 ng/ml) were observed. A highly significant positive correlation was found between OPN and body fat percentage ($r = 0.45$; $P < 0.0001$; Fig. 2). OPN levels were significantly correlated with several variables as shown in Table 2. However, after adjusting for body fat, only the associations of OPN with insulin, QUICKI, uric acid, vWF, CRP, alkaline phosphatase, and hepatic enzymes (ALT, AST, and γ -GT) remained statistically significant. The significantly elevated levels of hepatic enzymes exhibited by the obese patients suggest that these subjects may suffer from fatty liver or some other liver dysfunction influencing OPN concentrations. However, OPN levels were still associated with body fat percentage after adjusting for ALT ($r = 0.34$; $P = 0.003$). To analyze the relation or influence of bone mass with circulating OPN concentrations, bone densitometry was performed in a subset of 20 obese patients (five male and 15 female); none of them exhibited osteoporosis. No significant differences ($P = 0.993$) in OPN levels between patients with osteopenia ($n = 7$; lumbar spine BMD, 0.90 ± 0.05 g/cm²; OPN, 76.3 ± 38.5 ng/ml) and normal subjects ($n = 13$; BMD, 1.13 ± 0.10 g/cm²; OPN, 76.1 ± 28.1 ng/ml) were observed. OPN concentrations and lumbar spine BMD were not significantly related ($r = 0.27$; $P = 0.245$).

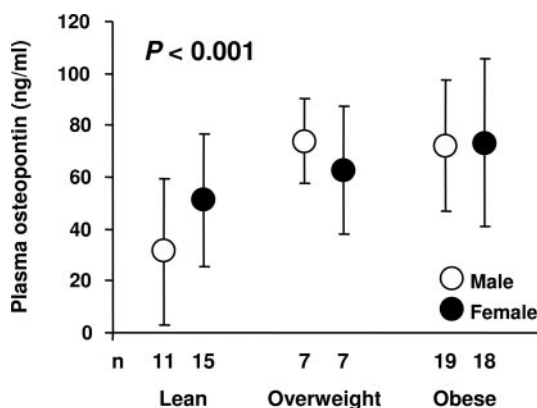


FIG. 1. Comparison of plasma OPN concentrations in the lean, overweight, and obese groups. Values are means \pm SD. Statistical differences were assessed by one-way ANOVA.

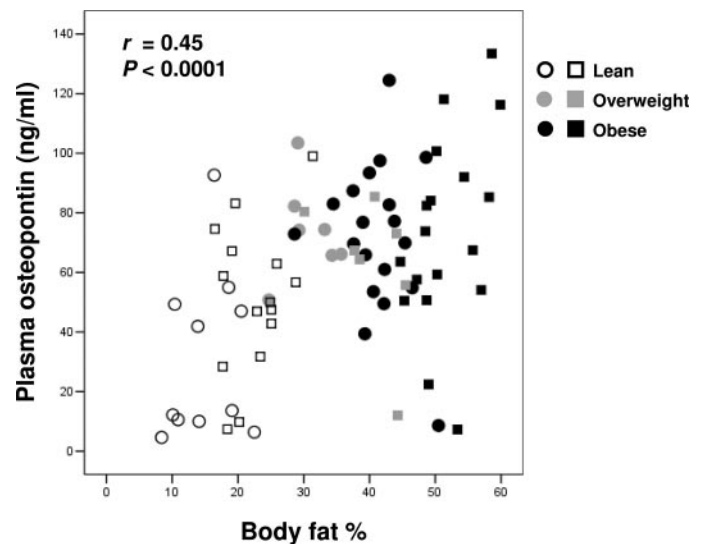


FIG. 2. Scatter diagram showing the highly significant positive correlation found between body fat percentage and circulating concentrations of OPN. Pearson's correlation coefficient and P value are indicated. Circles represent males, and squares stand for females.

Detection of OPN in adipose tissue and OPN expression in obesity

The presence of OPN protein in sections of omental adipose tissue was evaluated by immunohistological analysis (Fig. 3). Both adipocytes and cells of the stroma-vascular fraction were immunopositive for OPN (Fig. 3, B and E). As expected, CD68 staining in adipose tissue was localized only to macrophages (Fig. 3, C and F). OPN labeling was stronger in CD68-positive cells, but a marked staining was also observed in fully mature adipocytes. Adipose tissue mRNA expression of *OPN* and its relation to the adiposity excess characteristic of obesity were also analyzed. To gain insight into this condition, mRNA expression of *OPN* in omental adipose tissue was measured in two groups of obese women classified according to the absence or presence of T2DM and compared with that of weight-stable lean women. Obese women with T2DM showed higher glucose concentrations in the fasting state ($P < 0.05$) and 2 h after an OGTT ($P < 0.05$) than lean and obese NG individuals. Moreover, they showed a significant reduction ($P < 0.05$) in adiponectin concentrations in comparison with obese NG women (Table 3).

The mRNA expression of *OPN* was significantly increased in omental adipose tissue of obese NG women compared with lean individuals (2.44 ± 0.88 vs. 1.00 ± 0.52 arbitrary units, $P < 0.05$; Δ Ct, 5.04 ± 0.60 and 6.37 ± 0.71 , respectively; Fig. 4A) and further increased in obese T2DM women (8.00 ± 4.74 , $P < 0.05$ vs. both lean and obese NG; Δ Ct, 3.44 ± 0.88). *CD68* mRNA expression in adipose tissue showed a similar tendency to that exhibited by *OPN* expression (obese T2DM 3.06 ± 2.20 , obese NG 1.71 ± 0.72 , lean 1.00 ± 0.44 arbitrary units; Δ Ct 4.96 ± 1.13 , 5.59 ± 0.63 , and 6.37 ± 0.82 , respectively; Fig. 4B), but the differences fell out of statistical significance ($P = 0.091$). *OPN* and *CD68* mRNA were not significantly related ($r = 0.33$; $P = 0.351$). Expression of *OPN* in omental adipose tissue was significantly correlated with all variables analyzed except for age and plasma adiponectin concentrations (Table 4).

TABLE 2. Univariate analysis of the correlation between OPN and other variables, unadjusted and after adjusting for body fat

Variable	Plasma OPN			
	Unadjusted correlation		Adjusted correlation	
	r	P value	r	P value
Sex	0.05	0.671	−0.08	0.490
Age	0.18	0.119	−0.09	0.437
BMI	0.42	<0.0001	0.08	0.477
Body fat	0.45	<0.0001		
SBP	0.22	0.070	0.00	0.986
DBP	0.19	0.108	−0.01	0.952
Glucose	0.30	0.009	0.18	0.117
Insulin	0.46	<0.0001	0.29	0.010
QUICKI	−0.54	<0.0001	−0.36	0.001
Triglycerides	0.29	0.011	0.22	0.054
Cholesterol	0.34	0.003	0.19	0.111
LDL-cholesterol	0.24	0.035	0.03	0.786
HDL-cholesterol	−0.18	0.114	0.01	0.988
Uric acid	0.42	<0.001	0.25	0.033
Fibrinogen	0.48	<0.0001	0.24	0.052
vWF	0.46	<0.001	0.29	0.024
Homocysteine	0.27	0.031	0.11	0.376
CRP	0.52	<0.0001	0.30	0.013
TNF- α	0.22	0.060	0.06	0.627
ALT	0.43	<0.001	0.31	0.006
AST	0.40	<0.001	0.34	0.003
Alkaline phosphatase	0.42	<0.001	0.33	0.004
γ -GT	0.43	<0.0001	0.31	0.006
Creatinine	0.13	0.262	0.21	0.079
Adiponectin	−0.18	0.118	−0.10	0.377
Leptin	0.38	<0.001	−0.09	0.451

Values are Pearson's correlation coefficients and associated *P* values. CRP and leptin concentrations were logarithmically transformed for statistical analysis. DBP, Diastolic blood pressure; SBP, systolic blood pressure.

OPN concentrations after diet-induced weight loss

After an average period of 14 wk following the hypocaloric regimen, patients lost an average of 8.5 kg ($P = 0.002$), and

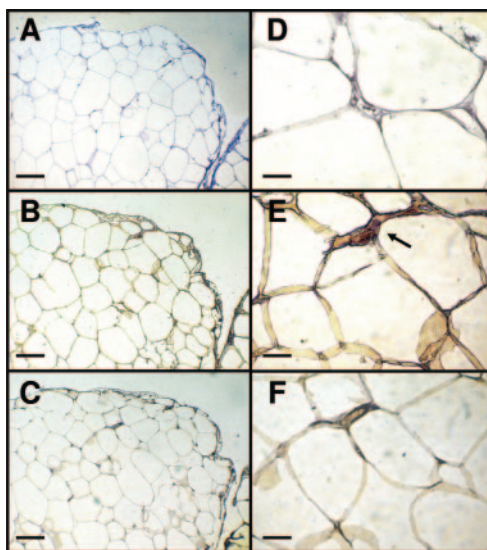


FIG. 3. Immunostaining of OPN and CD68 in human omental adipose tissue. Immunoreactivity for CD68 was localized to macrophages (C and F). Positivity (brown staining) was observed for OPN (B and E) in both fully mature adipocytes and cells of the stroma-vascular fraction, being stronger for CD68-positive cells (arrow). No immunoreactivity was found without any primary antibody (negative control, A and D). Magnification, $\times 100$ (A–C) (scale bar, 80 μ m) and $\times 400$ (D–F) (scale bar, 20 μ m).

their waist circumference decreased a mean of 12.6 cm ($P < 0.0001$) (Table 5). Significant decreases in BMI, body fat, and waist-to-hip ratio as well as a significant improvement in systolic ($P < 0.001$) and diastolic ($P = 0.004$) blood pressures were also observed. Glucose and insulin concentrations were within the normal ranges at baseline as well as after weight loss. Leptin concentrations, as expected after the reduction in body fat, were significantly reduced ($P < 0.001$). Diet-induced weight loss produced a statistically significant reduction in circulating OPN concentrations (Fig. 5), which decreased from 64.7 ± 22.1 to 36.6 ± 20.1 ng/ml ($P = 0.006$).

Discussion

The main findings of the present study are 1) that plasma OPN concentrations are increased in overweight and obese subjects, 2) that circulating concentrations of OPN correlate with body fat, 3) that OPN mRNA and protein are expressed in omental adipose tissue, 4) that the expression in this fat depot is increased in obesity and further elevated in obesity-associated T2DM, and 5) that modest diet-induced weight loss is accompanied by a significant decline in plasma OPN levels.

This is, to our knowledge, the first study describing increased plasma OPN levels in human obesity. Obese patients exhibited a 2-fold increase in plasma OPN concentrations compared with lean individuals. The significant positive correlation found in the present study between OPN and body fat seems to indicate that OPN levels are related to the adipose tissue amount. It is noteworthy that our data are the first to report that OPN is actually expressed by adipose

TABLE 3. Characteristics of lean and obese women included in the adipose tissue expression study

	Lean NG	Obese NG	Obese T2DM
n	4	4	4
Age (yr)	35.8 ± 8.8	38.8 ± 9.4	40.5 ± 6.7
BMI (kg/m ²)	20.7 ± 2.6	48.1 ± 5.2 ^a	55.0 ± 11.0 ^a
Body fat (%)	26.3 ± 6.2	56.3 ± 2.9 ^a	55.0 ± 4.0 ^a
Glucose (mmol/liter)	4.9 ± 0.5	5.1 ± 0.6	7.9 ± 1.2 ^{a,b}
2-h OGTT glucose (mmol/liter)	6.1 ± 1.4	6.6 ± 0.6	15.1 ± 6.1 ^{a,b}
Insulin (μU/ml)	7.5 ± 4.1	20.8 ± 10.0 ^a	26.3 ± 13.3 ^a
QUICKI	0.366 ± 0.042	0.309 ± 0.017 ^a	0.285 ± 0.023 ^a
CRP (mg/liter)	2.0 ± 1.2	6.3 ± 2.1 ^a	9.3 ± 3.3 ^a
ALT (IU/liter)	9 ± 2	33 ± 21 ^a	40 ± 21 ^a
Adiponectin (μg/ml)	11.6 ± 6.1	8.8 ± 1.9	5.1 ± 1.2 ^b
Leptin (ng/ml)	9.1 ± 4.3	57.2 ± 20.0 ^a	73.6 ± 49.4 ^a

Data are presented as mean ± SD. Differences between groups were analyzed by Kruskal-Wallis followed by Mann-Whitney *U* tests. To convert glucose to mg/dl, divide by 0.05551.

^a *P* < 0.05 vs. lean NG.

^b *P* < 0.05 vs. obese NG.

tissue. However, OPN is derived from many cellular types, and the partial contribution of any other organ to circulating OPN remains unknown (34). Expression of OPN has been

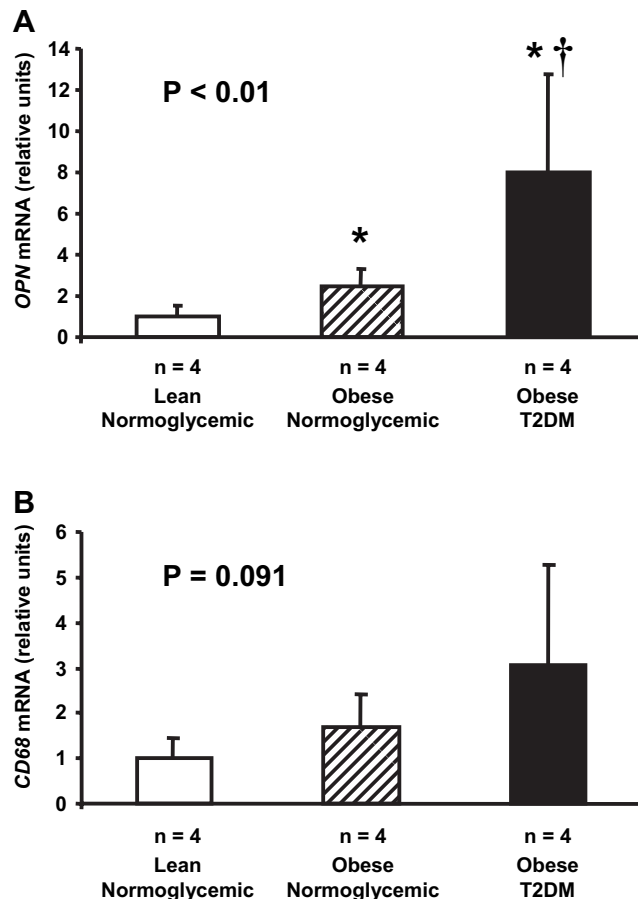


FIG. 4. Expression of *OPN* and *CD68* in omental adipose tissue from lean and obese women. PCR amplification of reverse-transcribed mRNA was performed on total RNA isolated from individual samples. Bars represent the mean ± SD of the ratio between *OPN* or *CD68* to *18S* rRNA from four lean normoglycemic, four obese normoglycemic, and four obese women with T2DM. The expression of *OPN* and *CD68* in lean subjects was assumed to be 1. Differences between groups were analyzed by Kruskal-Wallis followed by Mann-Whitney *U* tests. *, *P* < 0.05 vs. lean NG; †, *P* < 0.05 vs. obese NG.

observed, among others, in epithelial cells, macrophages, and atherosclerotic plaques (8). It has also been reported that *OPN* is expressed in adipose-derived stem cells (21). In the present study, we have demonstrated for the first time the presence of *OPN* by immunohistochemistry in human omental adipose tissue. *OPN* labeling was stronger in CD68-positive macrophages as might be expected, but a marked staining in fully mature adipocytes was evident. Furthermore, we have analyzed the mRNA expression of *OPN*, providing clear evidence that it is highly expressed in omental adipose tissue and significantly increased in obese patients compared with lean individuals. It is possible that part of the detected *OPN* mRNA is derived from infiltrated macrophages present in adipose tissue, which are further recruited by *OPN* itself in a vicious cycle (8, 35), but the immunohistochemical analysis shows that *OPN* is also expressed by adipocytes. Therefore, *OPN* secretion by omental adipose tissue may contribute to the increased circulating levels of *OPN* observed in obesity. Because *OPN* is expressed in both adipocytes and cells of the stroma-vascular fraction, the quantitative contribution of each cell type remains to be fully elucidated.

The expression of *OPN* is increased in the liver (20) and in the infarcted heart (18) of obese mice, which has been related to fibrosis and recruitment of inflammatory cells, respectively. Moreover, a direct stimulatory effect of leptin on the expression of *OPN* has been described to take place in mouse

TABLE 4. Univariate analysis of correlation between *OPN* mRNA in omental adipose tissue and other variables

Variable	<i>OPN</i> mRNA	
	ρ	<i>P</i> value
Age	0.29	0.359
BMI	0.86	<0.001
Body fat	0.71	0.009
Glucose	0.69	0.014
2-h OGTT glucose	0.74	0.006
Insulin	0.67	0.017
QUICKI	-0.83	<0.001
CRP	0.85	<0.001
ALT	0.75	0.005
Adiponectin	-0.40	0.223
Leptin	0.70	0.011

Values are Spearman's correlation coefficients (ρ) and associated *P* values.

TABLE 5. Effect of weight loss in obese male patients after a dietetic intervention

	Before weight loss	After weight loss	<i>P</i>
n	12	12	
Age (yr)	33.5 ± 15.1	33.8 ± 15.2	
Body weight (kg)	115 ± 20	106 ± 17	0.002
BMI (kg/m ²)	36.3 ± 5.1	33.6 ± 4.1	0.002
Body fat (%)	39.5 ± 8.9	36.2 ± 8.9	<0.001
Waist circumference (cm)	115 ± 15	103 ± 14	<0.0001
WHR	0.97 ± 0.07	0.92 ± 0.07	<0.0001
SBP (mm Hg)	140 ± 12	126 ± 13	<0.001
DBP (mm Hg)	94 ± 9	86 ± 10	0.004
Glucose (mmol/liter)	5.1 ± 0.6	5.2 ± 0.6	0.340
Insulin (μU/ml)	16.7 ± 7.8	14.7 ± 7.0	0.407
QUICKI	0.321 ± 0.026	0.324 ± 0.022	0.706
Leptin (ng/ml)	19.2 ± 7.8	13.0 ± 6.3	<0.001

Data are presented as mean ± SD. *P* values compare after *vs.* before weight loss by two-tailed paired Student's *t* tests. To convert glucose to mg/dl, divide by 0.05551. DBP, Diastolic blood pressure; SBP, systolic blood pressure; WHR, waist-to-hip ratio.

hepatocytes (20). This finding is in agreement with our data of high levels of OPN in the setting of hyperleptinemia. However, OPN concentrations were not significantly associated with leptin after adjusting for body fat.

The present study shows that increased body fat is accompanied by high concentrations of OPN in addition to high levels of CRP, TNF- α , and fibrinogen, thus reinforcing the observation that excess adiposity may contribute to the obesity-associated low-grade chronic inflammation with OPN emerging as an additional element of this condition. In this sense, a close association between OPN and CRP after adjusting for adiposity was found. The proinflammatory association between OPN and CRP is in agreement with previous findings (36, 37). Taken together, these associations may contribute, at least in part, to the obesity-associated inflammatory state, which takes place in the obesity-associated cardiovascular derangements. OPN also plays a role in atherosclerosis as a component of the extracellular matrix, a circulating adhesion molecule, and a chemoattractant cytokine. OPN transgenic mice developed larger atherosclerotic lesions with an atherogenic diet than nontransgenic mice (10), whereas OPN-deficient mice developed smaller atherosclerotic lesions than normal mice (38). Our data indicate that vWF concentrations are positively associated with circulating OPN levels. It is noteworthy that vWF levels have been suggested as an indirect indicator of thrombosis and atherosclerosis (39). In this sense, our data suggest that the

increased concentrations of OPN found in obesity may contribute to the increased atherosclerosis risk as well as to the endothelial dysfunction.

The increased levels of OPN found in obesity may be related to hyperglycemia and to the degree of insulin resistance. OPN concentrations were positively associated with insulin and negatively with QUICKI even after adjusting for body fat. High glucose levels have been shown to stimulate OPN expression through protein kinase C-dependent pathways as well as via the hexosamine pathway in cultured rat aortic smooth muscle cells (40). Moreover, OPN expression is repressed by insulin-sensitizing peroxisome proliferator-activated receptor- γ agonists (41). In this regard, expression of OPN in omental adipose tissue of obese women with T2DM was found to be increased in our study compared with obese NG women.

It has been shown that OPN modulates the expression and/or activity of proteins that regulate extracellular matrix remodeling. For example, OPN induces matrix metalloproteinase-2 (MMP-2) and MMP-9 activation (8, 19). Expansion of adipose tissue is a dynamic process in which matrix remodeling plays an important role (42, 43). Obesity is associated with increased expression of MMP-2 (43) and MMP-9 (44). Taken together, these observations suggest that OPN might be involved in the remodeling of the adipose tissue extracellular matrix that takes place in obesity. Plasma levels and expression in adipose tissue of another protein with functions related to OPN, SPARC/osteonectin, is similarly up-regulated in obesity (45, 46), and its adipose expression reportedly decreases after weight loss in obese patients (47). Furthermore, osteonectin-null mice exhibit increased adiposity without significant differences in overall body weight (48). In this sense, the analysis of adipose tissue metabolism in OPN-deficient mice, which also showed a normal body weight (49), deserves special attention.

OPN levels were strongly associated with hepatic enzymes (ALT, AST, and γ -GT), which are commonly increased in obese patients in relation to fatty liver. In this sense, OPN concentrations have been shown to be increased in patients with fulminant hepatic failure (50) as well as being involved in the development of nonalcoholic steatohepatitis in mice (20). The presence of fatty liver in the obese patients included in our study could underlie the obesity-associated increased

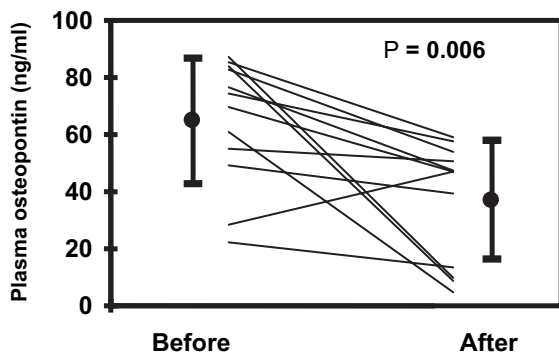


FIG. 5. Comparison of plasma concentrations of OPN determined in obese patients before and after weight loss after a dietetic intervention. Values are means ± SD. Statistical differences were assessed by two-tailed paired Student's *t* test.

OPN concentrations observed. However, after adjusting for ALT, a marker of fatty liver (51), OPN levels were still associated with body fat percentage. Furthermore, alkaline phosphatase was also positively associated with OPN concentrations. OPN is recognized by osteoblast cells and induces alkaline phosphatase activity, both proteins being expressed during osteoblastic differentiation. Reciprocally, OPN can be regulated by alkaline phosphatase (52).

Moderate weight loss has been shown to improve obesity-associated cardiovascular problems (53). Although the weight loss experienced by volunteers in our study was only modest, it was followed by a reduction in OPN concentrations, therefore indicating that plasma OPN appears to be very responsive to changes in energy balance. OPN is an established biomarker of cardiovascular events, being up-regulated in tissues during several pathological processes including atherosclerosis, valve stenosis, and myocardial infarction (54–57). Consequently, the increased levels observed in obesity herein may be associated with obesity-related cardiovascular derangements (17, 58). The reduction of OPN plasma concentrations observed in obese patients after weight loss may contribute to the improved cardiovascular risk profile. Therefore, a fall in OPN levels may participate in the decrease observed in cardiovascular morbidity after weight loss.

In conclusion, these results indicate that plasma OPN concentrations are increased in obese patients. In addition, mRNA expression of *OPN* in omental adipose tissue is up-regulated in obese volunteers being further increased in obesity-associated T2DM. Moreover, weight loss reduces OPN concentrations, which may contribute to the beneficial effects accompanying weight reduction. Additional longitudinal studies should allow us to determine the predictive value of this biomarker for obesity-associated cardiovascular diseases as well as for other comorbidities such as T2DM and non-alcoholic fatty liver disease.

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