

Gender Differences in Adiponectin and Leptin Expression in Epicardial and Subcutaneous Adipose Tissue. Findings in Patients Undergoing Cardiac Surgery

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Introduction and objectives. The high level of metabolic activity present in visceral adipose tissue is associated with the development of atherothrombosis. Subcutaneous adipose tissue secretes larger quantities of the adipocytokines leptin and adiponectin than visceral adipose tissue. Epicardial adipose tissue secretes inflammatory mediators, and the resulting proinflammatory activity is greater than that associated with subcutaneous adipose tissue. The aim of this study was to compare adiponectin and leptin expression in human epicardial and subcutaneous adipose tissue.

Methods. Samples of both epicardial and subcutaneous adipose tissue were taken from 46 patients who were undergoing heart surgery, coronary artery bypass surgery, or aortic or mitral valve replacement. Levels of protein and gene expression of leptin and adiponectin were assessed immunohistochemically and by RT-PCR, respectively. Levels of mRNA expression in the 2 adipose tissue types were compared by real-time quantitative PCR.

Results. Significant differences were found between adiponectin and leptin mRNA expression in epicardial and subcutaneous adipose tissue, with epicardial adipose tissue exhibiting lower levels of adiponectin and leptin expression. Moreover, adiponectin and leptin mRNA expression in epicardial adipose tissue was higher in women than men.

Conclusions. Adiponectin and leptin expression is lower in epicardial than in subcutaneous adipose tissue. Moreover, there are differences in adiponectin and leptin expression in epicardial adipose tissue between women and men.

Key words: *Adiponectin. Leptin. Epicardial adipose tissue.*

Influencia del sexo en la expresión de adiponectina y leptina en el tejido adiposo epicárdico y subcutáneo. Estudio en pacientes sometidos a cirugía cardiaca

Introducción y objetivos. La grasa visceral presenta una intensa actividad metabólica relacionada con el desarrollo de aterotrombosis. El tejido adiposo subcutáneo secreta en mayor cantidad las adipocitocinas leptina y adiponectina que el tejido adiposo visceral. El tejido adiposo epicárdico secreta mediadores inflamatorios; esta actividad proinflamatoria es mayor que la del tejido adiposo subcutáneo. En este trabajo tratamos de determinar y comparar la expresión de adiponectina y leptina en el tejido adiposo epicárdico y subcutáneo humano.

Métodos. Para el análisis se extrajo una muestra de tejido adiposo epicárdico y tejido adiposo subcutáneo de 46 pacientes en los que se había realizado cirugía cardiaca, *bypass* coronario o reemplazo valvular aórtico/mitral. La expresión proteica y génica de la adiponectina y la leptina fue analizada mediante inmunohistoquímica y RT-PCR, respectivamente. Los niveles de expresión de ARN mensajero se analizaron mediante la técnica comparativa de PCR en tiempo real, en ambos tejidos.

Resultados. Hay diferencias significativas de expresión de ARN mensajero de la adiponectina y la leptina entre el tejido adiposo epicárdico y subcutáneo, con una menor expresión de la adiponectina y la leptina en la grasa epicárdica. En comparación con los varones, las mujeres muestran una mayor expresión de adiponectina y leptina en tejido adiposo epicárdico.

Conclusiones. El tejido adiposo epicárdico expresa menos adiponectina y leptina que el subcutáneo. Hay una expresión diferencial entre varones y mujeres en cuanto a la expresión de adiponectina y leptina en el tejido adiposo epicárdico.

Palabras clave: *Adiponectina. Leptina. Tejido adiposo epicárdico.*

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ABBREVIATIONS

AT: adipose tissue
cDNA: complementary DNA
HDLc: high density lipoprotein cholesterol
mRNA: messenger RNA

INTRODUCTION

Adipocytokines are bioactive proteins that are synthesized and secreted by adipose tissue (AT). They play an important role in the regulation of energy metabolism, immune function, vascular remodeling, angiogenesis, and neuroendocrine function.¹ These proteins can act locally, through autocrine/paracrine mechanisms, or systemically, through endocrine effects.²

Adiponectin is a 28-kDa protein³ that is synthesized mainly by adipocytes. Its production by other cell types, such as cardiomyocytes, has also been reported.⁴ This hormone, which circulates in abundance in human plasma, constitutes 0.01% of total plasma proteins.⁵ One of the major roles of this hormone is to improve insulin resistance by increasing AT fatty acid oxidation and decreasing plasma fatty acid concentrations and intracellular triglyceride accumulation in the liver and muscle. It also plays a protective role in atherosclerotic processes,⁶ and its concentrations are reduced in states of insulin resistance, such as obesity and type 2 diabetes,^{7,8} as well as in patients with coronary artery disease.⁹

Leptin is a 16-kDa cytokine that acts as a satiety signal in the central nervous system and is related to glucose and insulin metabolism. It has been proposed to be the link between obesity, diabetes and cardiovascular risk.¹⁰

Serum leptin concentrations increase exponentially with the increase in body fat mass,¹¹ and hyperleptinemia is a reflection of the leptin resistance associated with obesity.¹² Patients with heart failure present elevated serum leptin concentrations,¹³ which have also been associated with a decrease in arterial distensibility¹⁴ and with increased inflammatory marker levels in obese individuals.¹⁵

Both adipocytokines are secreted in greater amounts by subcutaneous AT than by visceral AT.^{16,17} It has recently been demonstrated that epicardial AT releases higher proportions of different inflammatory mediators than subcutaneous AT in patients at high cardiac risk.¹⁸ Likewise, Iacobellis et al¹⁹ detected adiponectin protein expression in epicardial AT, observing lower levels in patients with severe coronary artery disease.¹⁹ This finding attributes new functions to epicardial AT, although the significance of the results has yet to be elucidated.

In view of previous studies indicating the antiinflammatory properties of adiponectin²⁰ and the association between leptin and inflammatory markers,¹⁵ we proposed to determine whether there was any difference between the epicardial and subcutaneous AT in patients with cardiovascular disease in terms of adiponectin and leptin messenger RNA (mRNA).

METHODS

This study was carried out with the approval of the local ethics committee and the informed consent of all the participating patients. Samples of epicardial and subcutaneous AT were obtained from 46 cardiac surgery patients, 15 women and 31 men, who had undergone coronary artery bypass surgery or aortic, or mitral valve replacement. The tissue was collected prior to cardiopulmonary bypass and was immediately frozen in liquid nitrogen and stored at -80°C until processing. As each patient served as his or her own control, only those with some type of viral infection were excluded.

The mean age of the women included was 71.1 (1.3) years, whereas that of the men was 67.4 (1.7) years, and the mean body mass index was 29.5 (1.3) and 28.48 (0.5), respectively. The clinical parameters and features assessed, as well as the medications received by the patients, are shown in Table.

Immunohistochemistry

The qualitative analysis of adiponectin and leptin protein expression was performed in a small portion of the epicardial and subcutaneous AT of a randomly chosen patient.

The tissue samples were fixed in 4% formaldehyde, dehydrated and cut into 5- μm sections.

Antigen detection consisted of incubation with the primary antibody. In the case of adiponectin, a 1/200 dilution of goat anti-ACRP30 N-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was employed overnight at 4°C . For the detection of leptin, the samples were incubated for one hour at room temperature with rabbit anti-Ob A20 antibody (Santa Cruz Biotechnology); the samples were subsequently incubated with the streptavidin-peroxidase complex Dako LSAB[®] System HRP (Dako) for 30 minutes and developed for 10 minutes with 3,3'-diaminobenzidine tetrahydrochloride (DAB), prepared according to the instructions of the manufacturer.

Negative controls were prepared with the primary antibodies, previously blocked overnight at 4°C with their respective antigens or using TBS without antibody.

RNA Extraction

RNA was extracted by the homogenization of the AT, under cold conditions, in a sodium citrate solution (0.025

TABLE 1. Main Clinical Features of the Study Patients and Medications They Received

Study Group (n=46)	Men (n=31)	Women (n=15)	Men Versus Women
Age, mean (SD), y	67.4 (1.7)	71.1 (1.3)	NS
BMI, mean (SD)	28.48 (0.5)	29.5 (1.3)	NS
Total cholesterol, mean (SD), mg/dL	174.4 (7)	197 (10.4)	NS
LDL-C, mean (SD), mg/dL	104.7 (7)	91.1 (13.5)	NS
HDL-C, mean (SD), mg/dL	38.5 (2.5)	34.6 (3.8)	NS
Triglycerides, mean (SD), mg/dL	131.1 (9.6)	131.8 (15.8)	NS
Hypertension	64%	73%	NS
Type 2 diabetes	35%	33%	NS
Ischemic heart disease	80%	60%	NS
Valve replacement	29%	53%	NS
Beta-blockers	31%	57%	NS
Aspirin	58.6%	64.3%	NS
Statins	27.6%	35.7%	NS
Clopidogrel	6.9%	7.1%	NS
Nitroglycerin	51.7%	42.9%	NS
Calcium antagonists	17.2%	28.6%	NS
Insulin	34.5%	28.6%	NS
Oral antidiabetic agents	13.8%	6.7%	NS

BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; NS: not significant; SD: standard deviation. The data are expressed as mean values plus or minus the standard deviation or as percentages of patients in the study group. There are no statistically significant differences between men and women for any of the parameters analyzed ($P > .05$ in every case).

mol/L) composed of guanidine thiocyanate (4 mol/L), beta-mercaptoethanol (0.1 mol/L) and sodium lauroyl sarcosinate (0.5%), followed by isolation using the method described by Chomzynski and Sacchi.²¹ The RNA concentration was analyzed at an absorbance of 260 nm and the purity of the sample was determined on the basis of the ratio of absorbance at 260 nm to absorbance at 280 nm. The sample was then treated with Dnase for two hours at 37°C, using 10 U/μL of DNase I and 20 U/μL of RNase inhibitor (both from Invitrogen Ltd., Paisley, UK) for every 5 μg of RNA. The proteins and the DNA were eliminated from the samples by extraction with phenol-chloroform-isoamyl alcohol. The RNA was precipitated with 96% ethanol and sodium acetate (0.3 mol/L) and resuspended in water treated with 0.1% diethylpyrocarbonate.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

First, 1.2 μg of purified RNA were utilized for the synthesis of complementary DNA (cDNA). The retrotranscription reaction was performed using 200 U of the enzyme MMLV reverse transcriptase (Invitrogen) in 20 μL of a solution composed of 20 mmol/L Tris-HCL buffer (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 1 mmol/L each dNTP, 20 U of ribonuclease inhibitor and

hexamer fragments as primers. The reaction conditions were: 50 minutes at 37°C, 10 minutes at 42°C and 5 minutes at 95°C.

The comparative analysis of adiponectin and leptin expression was carried out by means of real-time polymerase chain reaction. The technique was performed according to the instructions of the manufacturer, in 20 μL, using 2 μL of the cDNA obtained from retrotranscription, 0.25 μmol/L of the corresponding primers, 3 mmol/L MgCl₂, 2 μL SYBR Green (Roche Diagnostics SL, Barcelona, Spain) and 30 nmol/L Rox (Stratagene, La Jolla, CA, USA). Gene expression was quantified by means of the fluorescence emitted by the fluorochrome after the binding of SYBR Green to the double-stranded DNA. The fluorochrome Rox was utilized as a control for the DNA load in the reaction. A single peak per amplification product in the dissociation curve was considered to indicate purity.

The following primers were employed for the amplification of the adiponectin²² and leptin²² genes:

– Adiponectin

Sense: 5'-TGGTGAGAAGGGTGAGAA-3'

Antisense: 5'-AGATCTTGGTAAAGCGAATG-3'

– Leptin

Sense: 5'-TTGGCCCTATCTTTTCTATG-3'

Antisense: 5'-GCATACTGGTGAGGATCTGT-3'

As a control for the load and quality of the cDNA employed in the study, the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed²³:

– GAPDH

Sense: 5'-TCCATGACAACCTTTGGCATCGTGG-3'

Antisense: 5'-GTTGCTGTTGAAGTCACAGGA-GAC-3'

The gene amplification procedure consisted of denaturation at 95°C for five minutes, followed by 40 cycles involving denaturation (30 seconds at 95°C), hybridization (45 seconds at 6°C) and one minute at 72°C for DNA extension. The amplification products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized by means of the Typhoon 9410 image acquisition system (Amersham Pharmacia Biotechnology, Inc., Freiburg, Germany).

The analysis of the results obtained was carried out using the software installed in the real-time polymerase chain reaction system (Mx3000P™; Stratagene). For each sample, the software package calculates the number of the cycle in which the reader begins to detect a significant increase in fluorescence with respect to the baseline signal. This cycle is referred to as the cut-off point or threshold cycle. In each sample, the levels of adiponectin and leptin expression were normalized to the expression of the internal control, GAPDH.

Statistical Analysis

The results obtained in this study were expressed as mean value plus or minus the standard deviation (SD). The comparisons between epicardial and subcutaneous tissue from each patient were performed using the paired Student *t* test. For gender comparisons, unpaired Student *t* test was employed and the 95% confidence interval was calculated. *P* values less than .05 were considered to indicate statistical significance. For the statistical analysis, the GraphPad InStat software package was used.

RESULTS

The main clinical features of the 46 patients studies are shown in Table There were no significant differences between men and women in terms of the parameters analyzed. This Table also indicates the number of patients (men and women) receiving the different drug therapies employed in the treatment of cardiovascular diseases and diabetes and, again, the absence of statistically significant differences between the two groups. Only one man was being treated with glitazone (rosiglitazone).

Epicardial and subcutaneous AT express adiponectin and leptin (Figure 1). Amplification products of 221 bp, 223 bp and 376 bp were obtained for adiponectin, leptin,

and GAPDH, respectively. The corresponding negative controls showed no evidence of contamination.

Immunohistochemical analysis of protein expression revealed that the cytoplasm of the adipocytes in epicardial and subcutaneous AT is immunoreactive for adiponectin, and leptin. There was no staining whatsoever of the corresponding negative controls (Figure 1).

The relative values of the level of expression of adiponectin were higher than those of leptin in both epicardial and subcutaneous AT; however, the levels of adiponectin and leptin mRNA were significantly higher in subcutaneous AT than in epicardial AT. The adiponectin mRNA concentrations were significantly higher in subcutaneous AT (13.54 [0.59] [n=46] versus 12.3 [0.48] [n=46]; *P*<.05). Likewise, the values obtained for leptin were also significantly higher in subcutaneous AT (9.8 [0.39] [n=46] vs 8.7 [0.26] [n=46]; *P*<.01) (Figure 2).

There was no significant correlation between the clinical features shown in Table (age, body mass index, cholesterol levels, etc.) and the levels of adiponectin or leptin expression. When the levels of adiponectin and leptin expression were analyzed according to sex, we observed statistically significant differences. In the women, the degrees of adiponectin expression in epicardial AT were significantly higher than in the men (14.81 [0.47] [n=15] versus 11.08 [0.55] [n=31]; *P*<.001) (Figure 3A), whereas there were no differences in subcutaneous AT. The levels of expression of leptin were also higher in epicardial AT of women than in that of men (9.4 [0.4] [n=15] vs 8.3 [0.3] [n=31]; *P*<.05) (Figure 3B), whereas no differences were observed in subcutaneous AT.

In men, the differences between epicardial and subcutaneous tissues, which were statistically significant, were maintained both for adiponectin (11.28 [0.59] vs 12.83 [0.61]; *P*<.01) and for leptin (8.32 [0.33] vs 9.32 [0.36]; *P*<.01). However, significant differences were not observed between epicardial and subcutaneous AT in women (Figures 3A and 3B).

The ratio of adiponectin mRNA expression to leptin mRNA expression in epicardial AT was significantly higher in women than in men (1.62 [0.1] vs 1.35 [0.04]; *P*<.01) (Figure 4). As in the results reported above, there were no significant differences in subcutaneous AT (Figure 4).

No statistically significant differences were observed when the adiponectin and leptin mRNA expression in epicardial AT in patients who had undergone coronary bypass (vascular disease) was compared with that of patients who had undergone valve replacement (Figure 5).

DISCUSSION

Obesity is a cardiovascular risk factor that not only acts passively by increasing the workload of the heart,

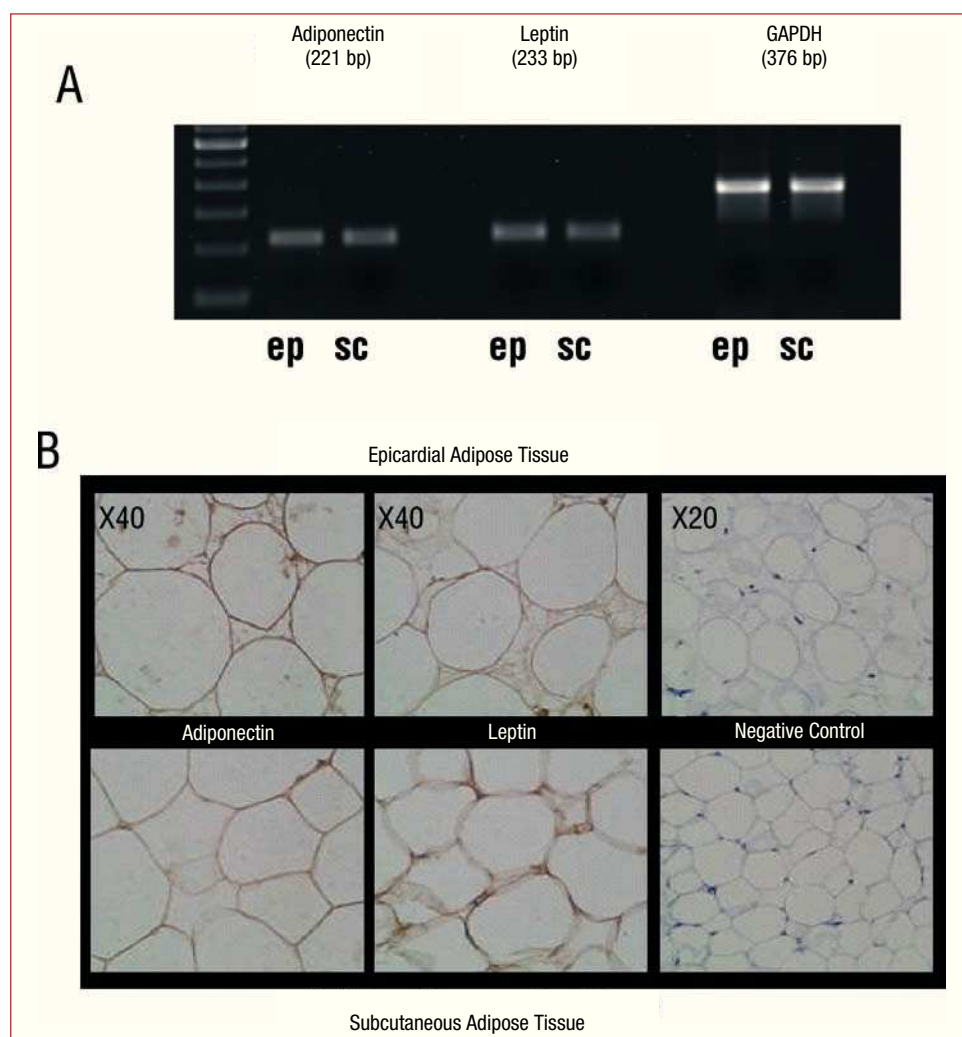


Figure 1. A: results of the reverse transcription polymerase chain reaction products in agarose gel for adiponectin, leptin and GAPDH in epicardial (ep) and subcutaneous (sc) adipose tissue. B: immunoreactivity of epicardial and subcutaneous adipose tissue of a patient with antibodies to adiponectin (left) and to leptin (center). The image on the right shows the negative controls.

but actively as well, by provoking changes in the plasma concentrations of certain molecular factors produced by AT. These factors have cardiovascular effects, as well as a regulatory role in energy metabolism,²⁴⁻²⁶ circumstances that convert fat into an authentic endocrine organ.

Adiponectin and leptin are among the factors secreted by AT and are included in the group of adipocytokines.¹ There is an association between plasma leptin concentrations and the classic cardiovascular risk factors, insulin resistance, the presence of metabolic syndrome and inflammatory markers, even when the proportion of body fat is within normal range.²⁷⁻²⁹ Plasma leptin concentrations are increased in hypercholesterolemic men, and are associated with higher coronary risk.³⁰

The hormone, adiponectin, is directly related to insulin sensitivity, abdominal obesity and changes in the lipid profile (particularly, low levels of high-density lipoprotein cholesterol [HDL-C]). On the other hand, there is an inverse relationship between vascular inflammation and the plasma levels of this hormone.²⁰ Moreover, a direct

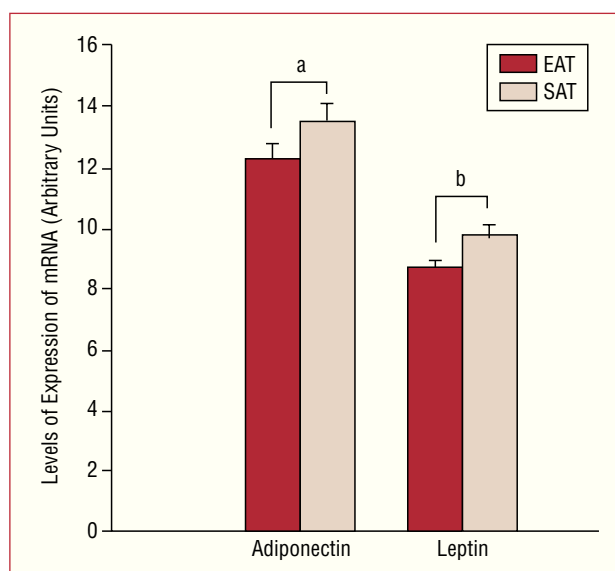


Figure 2. Results of quantitative real-time reverse transcription polymerase chain reaction showing the levels of adiponectin and leptin expression in epicardial adipose tissue (EAT) and subcutaneous adipose tissue (SAT) of 46 patients with respect to GAPDH. The superscripts indicate the statistically significant differences (^a $P < .05$; ^b $P < .01$).

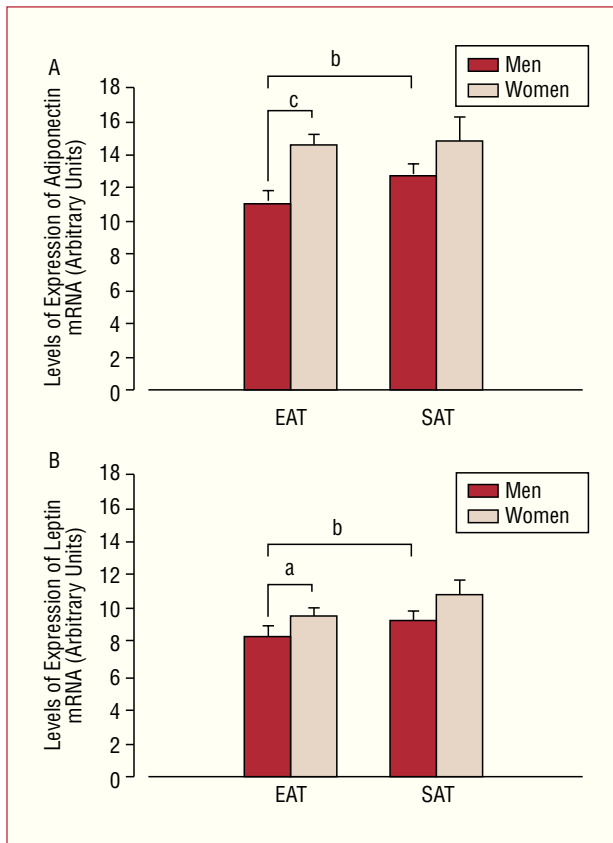


Figure 3. Results of quantitative real-time reverse transcription polymerase chain reaction showing levels of adiponectin (A) and leptin (B) expression in epicardial adipose tissue (EAT) and subcutaneous adipose tissue (SAT) of 31 men and 15 women, who had undergone cardiac surgery, with respect to GAPDH. The superscripts indicate the statistically significant differences (^a $P < .05$; ^b $P < .01$; ^c $P < .001$).

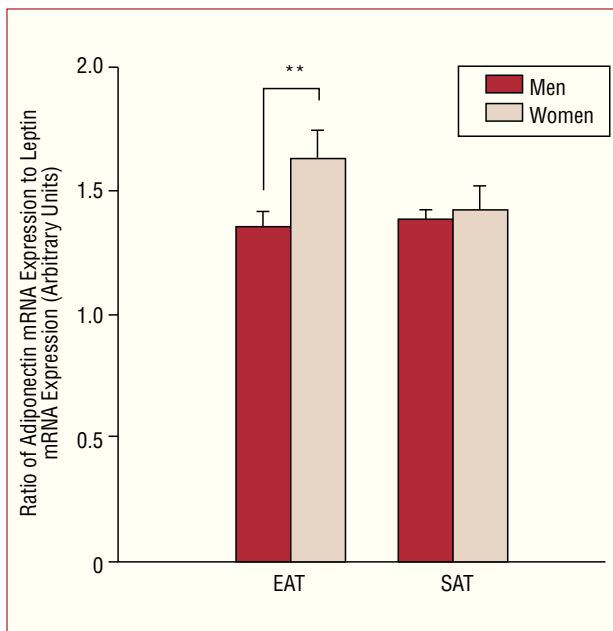


Figure 4. Ratio of adiponectin mRNA expression to leptin mRNA expression in epicardial adipose tissue (EAT) and subcutaneous adipose tissue (SAT) in men and women. ^{**} $P < .01$.

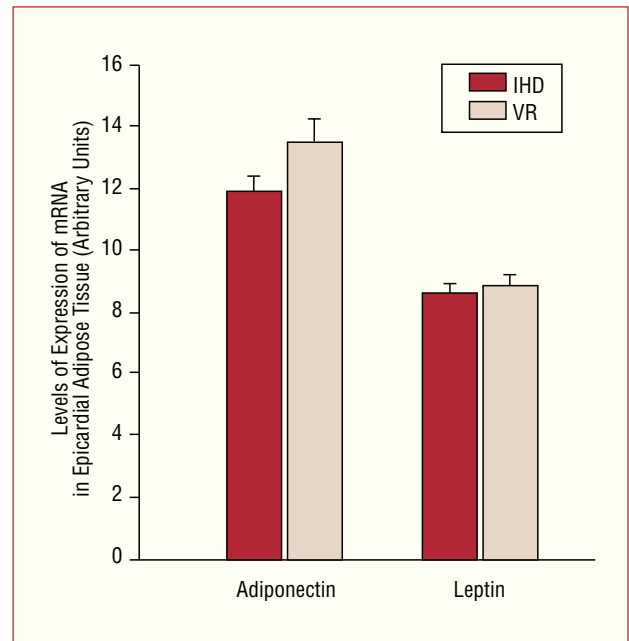


Figure 5. Results of quantitative real-time reverse transcription polymerase chain reaction showing adiponectin and leptin expression in epicardial adipose tissue, from 34 patients with ischemic heart disease (IHD) and 17 who underwent valve replacement (VR), with respect to GAPDH.

relationship between reduced plasma adiponectin concentrations and the extension of atherosclerosis has been observed in a number of studies, and lower levels of this hormone have been documented in men with ischemic heart disease.²⁵

According to a recent report, patients with ischemic heart disease present higher levels of expression of monocyte chemotactic protein (MCP-1) and of certain proinflammatory cytokines (interleukin 1) in epicardial AT as compared to subcutaneous AT,¹⁸ and it has been speculated that epicardial fat may play a paracrine role in diseases affecting the coronary arteries (atherothrombosis) and the myocardium (cardiac dysfunction).

Along this line, and given the association between leptin and proinflammatory markers, it could be expected that the level of expression of this hormone would also be higher in epicardial AT than in subcutaneous AT. In this respect, differences in leptin and adiponectin expression have been observed in different fat deposits, being more marked in subcutaneous AT than in visceral AT.^{16,17,31,32}

This report shows that epicardial and subcutaneous AT produce different adiponectin and leptin concentrations, which are significantly higher in subcutaneous AT. This difference is mainly due to the influence of gender; when the results are analyzed according to the sex of the patients, the epicardial fat of the women was found to produce higher concentrations

of adiponectin and leptin than that of the men (Figures 3A and 3B). Due to the fact that, in general, the level of adiponectin expression in AT is higher than that of leptin, the differences between epicardial and subcutaneous AT in terms of the expression of these hormones are more marked with respect to adiponectin than those observed with leptin. This finding could indicate that the adiponectin produced by AT plays a more relevant metabolic role than leptin.

In contrast to the results obtained by Iacobellis et al,¹⁹ who observed, in a group of 16 patients, that the epicardial AT of those with ischemic heart disease synthesized less adiponectin than that of the controls (aortic or mitral valve replacement), in our study, we found no significant differences between patients with ischemic heart disease (n=34) and those who underwent valve replacement (n=17) in terms of adiponectin expression. The lower levels of adiponectin and leptin expression in epicardial AT of the patients with ischemic heart disease did not reach statistical significance.

However, we can not rule out the possibility that the discrepancy encountered with respect to the data reported by Iacobellis et al be due to mechanisms of posttranscriptional regulation, which would explain the fact that no differences are observed in the levels of gene expression, and to differences in the characteristics of the patients, although not in the sample size, as we include a greater number of cases.

With respect to the results published by Baker et al,³³ we also observed a lower level of expression of adiponectin and leptin mRNA in epicardial AT as compared to peripheral AT (subcutaneous, omental, and perimuscular).³³ However, we consider the methodological validity of our approach to be greater since we employed both epicardial and subcutaneous AT from each patient, whereas Baker et al analyzed epicardial AT from patients who had undergone coronary artery surgery, comparing it with peripheral AT from other patients with no coronary artery disease or type 2 diabetes.³³ Thus, they were unable to determine whether there were differences in the expression of these hormones in epicardial and peripheral fat from the same patient.

On the other hand, we can not rule out the possibility that a portion of the adipocytokine production be due to the presence of inflammatory cells in the AT, although adiponectin and leptin are produced mainly by the adipocytes, whereas macrophages are more specialized in the production of cytokines, such as tumor necrosis factor alpha or interleukin 6.³⁴ Although 10% of the content of AT is composed of macrophages, the number of which is directly correlated with adiposity and the size of the adipocytes, there is no difference in the percentages of these inflammatory cells in subcutaneous and visceral AT. Thus, they can not be expected to influence the differences observed in our study.³⁵

A number of factors could explain our findings with respect to the differences between men and women. The adiponectin concentrations may be lower in men due to the effect of androgens.³⁶ Tsou et al³⁷ described the influence of sex hormones on plasma adiponectin levels, and the plasma leptin levels are also influenced by sex hormones.³⁸ This relationship of adiponectin and leptin to androgens and estrogens may, in part, have determined our data, particularly the lower level of adiponectin activity in epicardial AT in men.

Our results may have certain clinical relevance. As we mentioned above, there is a relationship between the low adiponectin concentrations and factors directly implicated in atherothrombotic vascular disease, and a direct relationship between plasma adiponectin concentrations and the risk for a number of vascular diseases, in particular between decreased levels of this hormone and the risk of myocardial infarction.³⁹ In this respect, the higher risk for vascular diseases of atherothrombotic etiology and, in particular, supposedly ischemic heart disease in men, could be related to the lower adiponectin production in epicardial AT, since, as we mentioned above, it could exert a direct paracrine effect on the coronary arteries, which would help to explain, in part, the differences in risk for atherothrombosis-related clinical conditions between men and women.

This possible pathophysiological explanation of the relationship between the metabolic activity of epicardial fat and the risk of coronary atherothrombosis is reinforced if we take into account the fact that the greatest proportion of epicardial fat is distributed in the epicardial coronary arteries.

CONCLUSIONS

In conclusion, we report for the first time that the production of leptin and adiponectin by epicardial AT is lower than that of subcutaneous AT. However, when the results are analyzed according to sex, a difference is observed in the expression of these hormones in epicardial AT, with a higher production in women than in men. The analysis of the ratio of adiponectin to leptin confirms that the difference in adiponectin expression in epicardial AT is related to sex, rather than to the presence of some type of inflammatory change, as could be observed after the comparison of the expression of these hormones in patients with ischemic heart disease and valve replacement.

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