CLINICAL STUDY

Plasma soluble tumor necrosis factor alpha receptors and leptin levels in normal-weight and obese women: effect of adiposity and diabetes

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Abstract

Objective: To explore the determinants of the tumor necrosis factor alpha (TNFα) system and their relationship with plasma leptin levels.

Methods: We studied a cohort of 157 diabetic and non-diabetic females with a wide range of adiposity distributed into five groups: control – body mass index (BMI) between 19 and 27 kg/m² (n = 24); obese – BMI between 27 and 40 kg/m² (n = 63); obese type 2 diabetes mellitus – BMI between 27 and 40 kg/m² with diabetes mellitus (n = 19); morbid obese – BMI >40 kg/m² (n = 29); and morbid obese type 2 diabetes mellitus – BMI >40 kg/m² with diabetes (n = 22). Fasting glucose levels, plasma total triglycerides and cholesterol, high-, low- and very low-density lipoprotein cholesterol were assayed by enzymatic and colorimetric methods. Plasma TNFα levels were measured by ELISA assay and insulin and leptin levels by radioimmunoenzymatic assays. Both soluble TNFα (sTNFα) receptors were measured by immunoenzymometric assays.

Results: All groups of patients showed significant increases in both sTNFα receptors relative to control. sTNFα receptor 1 (sTNFR1) was higher in morbid obese diabetic individuals compared with their non-diabetic counterparts (P < 0.003), while sTNFR2 was significantly different between obese and morbid obese subjects (P = 0.036). Bivariate correlation analysis showed a significant relationship between both plasma sTNFα receptors and BMI, percentage of body fat, fasting glucose, insulin and leptin. In multivariate analysis, both sTNF receptor plasma levels were predicted by percentage of body fat and the presence of diabetes (R² = 0.20 for sTNFR1 and sTNFR2). When plasma leptin levels were added into the model, this protein and the presence of diabetes explained 27% of the variance of the plasma sTNFR1 levels.

Conclusion: The presence of diabetes, adiposity or leptin levels are independent determinants of both sTNFα receptors. The independent association between plasma TNFα receptors and leptin levels in obese patients is consistent with the hypothesis that these proteins could be involved in the same pathway that regulates body adiposity.

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Introduction

Tumor necrosis factor alpha (TNFα), a cytokine mainly produced by immunological cells, has been implicated in the regulation of energy balance in different pathological conditions such as cancer (1), AIDS (2, 3) and other catabolic situations (4). This cytokine is also produced by adipose (5) and muscle tissues (6), and has recently been involved in the pathophysiology of obesity (7–9).

TNFα expression is increased in most of the rodent models of obesity examined to date (5). This increase in the adipose and muscle tissue TNFα expression has also been reported in obese people (6, 10) together with significant relationships between TNFα expression (or plasma protein product concentrations) and adiposity (11, 12). However, a considerable number of investigators have been unable to observe higher TNFα levels in obese compared with lean individuals (13).

Because the levels of soluble TNFα (sTNFα) receptors have been considered a reflection of the activity of the TNFα system (14), different studies have attempted to evaluate the effect of adiposity on these proteins and their findings have been controversial. Some small-sized studies have shown an increase in both soluble receptors (12, 13) or only in sTNFα receptor 2 (sTNFR2) (15–17) in obese subjects compared with controls. Furthermore, in a recent report developed in a large sample, no significant differences in both
plasma \(\text{sTNF} \alpha\) receptors were observed between lean and overweight men (18).

The expression and levels of these molecules have been related to glucose homeostasis (18), degree of hyperinsulinemia (15) or leptinemia (12), suggesting that the \(\text{TNF} \alpha\) system plays a central role in the insulin resistance associated with obesity, but the effect in obese people of insulin sensitivity or the presence of diabetes on both plasma \(\text{sTNF} \alpha\) receptors has not been well studied to date.

We report here the largest study to date performed on a group of diabetic and non-diabetic females with a wide range of adiposity, with the aim of exploring the factors that determine the \(\text{TNF} \alpha\) soluble receptor plasma levels, including age, adiposity, degree of hyperinsulinemia or leptinemia and the presence of diabetes.

Materials and methods

Subjects

We studied 157 females aged between 19 and 65 years with a wide range of adiposity and who were consecutively recruited from among those attending the outpatient clinics of the Hospital Universitari de Sant Joan de Reus, Spain. Four patient-group assignments, with respect to body mass index (BMI) and presence/absence of diabetes, were compared with a control group of healthy subjects with a BMI between 19 and 27 kg/m\(^2\). The subjects were defined as obese when the BMI was between 27 and 40 kg/m\(^2\) and as morbid obese when the BMI was \(>40\) kg/m\(^2\) according to the Spanish Consensus for obesity diagnosis and treatment (19). The presence of diabetes was confirmed when a previous diagnosis of non insulin-dependent diabetes mellitus (NIDDM) had been performed or when a fasting plasma glucose of \(>7\) mmol/l was observed on two consecutive occasions. Glycated hemoglobin \(\geq 7.0\) was considered as diabetes under poor metabolic control (20). Eighty-four patients were premenopausal and 73 were post-menopausal females. The characteristics of all the study subjects are summarized in Tables 1 and 2. Exclusion criteria were the presence of infectious, inflammatory, neoplastic or systemic diseases, hypothyroidism or endocrine diseases other than diabetes, or the active use of anti-obesity drugs. The study protocol was approved by the hospital ethics committee and all subjects gave informed written consent to their participation in the study.

Data collection and laboratory procedures

Height was measured without shoes to the nearest 0.5 cm. Body weight was measured to the nearest 0.1 kg (with the subject lightly clothed) and BMI was calculated as weight/height\(^2\). Waist circumference was measured midway between the lower rib margin and the iliac crest. Hip circumference was determined as the widest circumference measured over the greater trochanter. The waist-to-hip ratio (WHR) was then calculated. Fat free mass (FFM) was assessed as previously described by tetrapolar bioelectrical impedance at 50 kHz (Human-In ScanR, Dietosystem, Madrid, Spain) using the gender and fat-specific equations validated by Segal et al. (21). Fat mass (FM) was then calculated as the difference between total body weight and FFM.

Fasting blood samples were collected in the morning between 0800 h and 1000 h. Plasma and serum were separated immediately by centrifugation and aliquots were frozen at \(-80^\circ \text{C}\) for subsequent batched analysis. Plasma total triglycerides and cholesterol, high-, low- and very low-density lipoprotein cholesterol (HDL, LDL and VLDL cholesterol) and glucose levels were assayed by the hospital’s routine chemistry laboratory. A commercial RIA kit was used to determine fasting plasma insulin (Amersham, Little Chalfont, Bucks, UK). The lowest limit of detection was 48 pg/ml. The intra- and inter-assay coefficients of variation for insulin were 5.05% and 13.4% respectively. Homeostasis model assessment of insulin resistance (HOMA IR) was then calculated as previously described (12):

\[
\text{HOMA IR} = \frac{\text{fasting insulin (U/ml)} \times \text{fasting glucose (mmol/L)}}{22.5}
\]

Immunoenzymometric assays were used to determine the levels of \(\text{sTNFR}1\) and \(\text{sTNFR}2\) concentrations in

<table>
<thead>
<tr>
<th>Table 1 Anthropometric and biometric characteristics of the study subjects. Values are expressed as means (S.E.M.).</th>
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<tbody>
<tr>
<td>Control (n = 24)</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Height (cm)</td>
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<tr>
<td>BMI (kg/m(^2))</td>
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<tr>
<td>Body fat (%)</td>
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<tr>
<td>Waist (cm)</td>
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<tr>
<td>WHR</td>
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Comparisons were performed using one-way ANOVA with multiple comparisons. *\(P < 0.05\), **\(P < 0.001\) vs control; †\(P < 0.05\), ‡\(P < 0.001\) vs corresponding non-diabetic group; §\(P < 0.05\), §§\(P < 0.001\) vs obese or obese type 2-diabetes mellitus.
plasma (BioSource, Fleunes, Belgium). The minimum detectable concentration was estimated to be 50 pg/ml and 0.1 ng/ml for each of the two receptors respectively. The intra-and inter-asssay coefficients of variation were <6.5% and <8.9% respectively for sTNFR1 and <3.3% and <6.9% respectively for sTNFR2. TNF levels were measured by ELISA (Pharmingen, San Diego, CA, USA). The intra- and inter-assay coefficients of variation were <5.8% and <13.8% respectively, and the limit of sensitivity was 4 pg/ml. Plasma leptin levels were measured by a radioimmunoenzymatic assay using commercial kits (Linco Research, St Louis, MO, USA). Within- and between-assay variations were 4.98% and 4.5% respectively and the limit of sensitivity was 0.5 ng/ml.

Statistical methods

Descriptive results of continuous variables are expressed as means±S.E.M. The variables with non-gaussian distributions, such as leptin and insulin levels, were logarithmically transformed to approach normal distribution. The means are expressed as geometric means. One-way ANOVA with multiple comparisons was used to explore differences between groups. Relationships between two quantitative variables were assessed by Pearson’s correlation coefficient. A stepwise multiple regression analysis was performed to identify the factors affecting plasma levels of sTNFα receptors. Age, percentage of fat mass, WHR, fasting plasma glucose and insulin concentrations, and presence/absence of diabetes mellitus were entered as independent variables. The model R^2 indicates the percentage variance in the dependent variable that is explained by the independent variables included in the model. Plasma leptin levels were introduced in a second model exploring the determinants of both sTNFα receptors. All statistical calculations were performed using the SPSS/PC software. Values were considered statistically significant with P < 0.05.

Results

Table 1 contains the anthropometric characteristics of the study groups. BMI, percentage of body fat and waist circumference increased in relation to the degree of obesity. No significant differences in anthropometric variables were observed in relation to the presence or absence of diabetes except a higher WHR in diabetic patients relative to non-diabetic patients. Diabetic patients were treated either by diet alone (n = 28) or by oral anti-diabetic agents (n = 11). Only one diabetic patient required insulin at the time of the study. Glycemic control (Hba1c <7.0%) was good in 14 patients (12 patients were put on a diet and two were treated with metformin) and poor in 25 patients (16 were put on a diet, five were treated with sulfonylurea and four were treated with metformin). None of the patients were taking thiazolidenedione during the study. Fasting glucose levels were significantly increased in patients with diabetes mellitus compared with controls and their obese counterparts. Plasma total cholesterol, HDL cholesterol, VLDL cholesterol and triglycerides were increased in all groups of patients compared with controls. Increases in plasma leptin and insulin levels were observed in morbid obese patients in relation to obese and control subjects (Table 2). No significant differences were observed between groups in relation to plasma TNFα levels.

All groups of patients showed a significant increase in both plasma sTNFα receptors compared with control (P < 0.001), even after the data had been adjusted for age (data not shown). As shown in Fig. 1, sTNFR1 was higher in morbid obese diabetic individuals compared with obese (P < 0.001) or morbid obese non-diabetic patients (P = 0.003). In relation to the sTNFR2, a significant mean difference was observed between the obese and the morbid obese patients (P = 0.036), obese type 2 diabetic group (P = 0.006), and morbid obese type 2 diabetic group (P < 0.001). Morbid obese diabetic patients showed higher sTNFR2 levels than
their non-diabetic counterparts, although the differences were not statistically significant ($P = 0.065$).

After adjusting for adiposity, both sTNFα receptors were higher in obese diabetic patients ($n = 41$; $2.11 \pm 0.09$ ng/ml for sTNFRI and $5.66 \pm 0.26$ ng/ml for sTNFRII) than non-diabetic obese patients ($1.85 \pm 0.06$ ng/ml for sTNFRI and $4.75 \pm 0.17$ ng/ml for sTNFRII; $P = 0.016$ and $P = 0.006$ respectively). No significant differences were observed in plasma leptin levels or either sTNFα receptor levels between patients with good or poor glycemic control or between patients who had been put on a diet and those who were treated with oral hypoglycemic agents, even after adjusting for differences in adiposity (data not shown). Neither did the type of antihypertensive therapy lead to any significant differences in sTNFα receptors, plasma insulin levels or HOMA IR. No significant differences were observed in leptin or sTNFα receptor levels between pre- and post-menopausal patients, after data had been adjusted for the percentage of body fat and age (data not shown). Bivariate correlation analysis showed a positive relationship between both plasma sTNFα receptors and BMI, percentage of total body fat, fasting glucose, glycated hemoglobin, HOMA IR, leptin (Fig. 2) and insulin; and a negative correlation with HDL cholesterol levels (Table 3). We also observed a significant correlation between levels of sTNFRII and WHR (Table 3). By contrast, no significant relationship was observed between plasma TNFα levels and any of the quantitative variables measured.

Finally, we performed a multiple linear regression in which plasma sTNFRI and sTNFRII were introduced as dependent variables, while age, percentage of body fat, WHR, fasting insulin and the presence of diabetes were the independent variables. Both plasma sTNFα receptor levels were significantly predicted by the percentage of body fat and the presence or absence of diabetes ($R^2 = 0.20$ and $R^2 = 0.19$, $P < 0.001$ for sTNFRI and sTNFRII). When plasma leptin levels were added to the previous model, the significant predictors of sTNFRI and sTNFRII were leptin levels and the presence of diabetes ($R^2 = 0.27$, $R^2 = 0.23$, $P < 0.001$ respectively). Similar results were obtained when only obese patients were analyzed (data not shown).

**Discussion**

Increasingly in the literature, there has been the suggestion that TNFα could be involved in the pathogenesis of insulin resistance associated with obesity. However, there has not been any consensus and several investigators have failed to demonstrate higher circulating levels of this cytokine, in this metabolic situation, compared with controls (13, 22). Similarly, in our study we did not observe any significant differences in levels of this cytokine between the obese patients and control subjects. The paracrine mechanism of TNFα secretion, the highly labile nature of this protein and the methodological difficulties of its quantification could be among the factors that may explain this.

![Figure 1](https://www.eje.org)
The assessment of the TNFα system activity based on the plasma TNFα receptor levels appears to be more reliable and of greater interest than plasma TNFα concentration itself. These proteins are easily detectable in plasma and appear to act as a buffer system prolonging the biological effects of TNFα (14, 23) and seem to reflect more accurately the degree of TNFα system activation than the concentrations of circulating TNFα (23).

Thus, we have observed significantly higher levels of both sTNFα receptors in obese, and especially in morbid obese, subjects compared with normal-weight subjects. Even when there is a general consensus in the increased levels of sTNFR2 in obesity (12, 13, 15–17), only a few studies have reported an increase in the sTNFR1 in obese patients compared with lean healthy controls (12, 13). A possible explanation for these discrepancies could rely on the limited sample size of some of these previous studies (15, 17). It is interesting to note that in the largest sample of individuals reported in the literature to date (n = 178) no significant differences have been observed in either

<table>
<thead>
<tr>
<th></th>
<th>Leptin (ng/ml)</th>
<th>sTNFR1 (ng/ml)</th>
<th>sTNFR2 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg/cm²)</td>
<td>0.738**</td>
<td>0.416**</td>
<td>0.356**</td>
</tr>
<tr>
<td>WHR</td>
<td>ns</td>
<td>ns</td>
<td>0.187*</td>
</tr>
<tr>
<td>Total body fat (%)</td>
<td>0.724**</td>
<td>0.406**</td>
<td>0.356**</td>
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<tr>
<td>Fasting glucose (mmol/l)</td>
<td>ns</td>
<td>0.204*</td>
<td>0.301**</td>
</tr>
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<td>HGe1c (%)</td>
<td>ns</td>
<td>0.240*</td>
<td>0.437**</td>
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<td>Plasma cholesterol (mmol/l)</td>
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<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>-0.310**</td>
<td>-0.290**</td>
<td>-0.328**</td>
</tr>
<tr>
<td>VLDL cholesterol (mmol/l)</td>
<td>0.187*</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.178*</td>
<td>ns</td>
<td>0.263**</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>-</td>
<td>0.492**</td>
<td>0.372**</td>
</tr>
<tr>
<td>Insulin (µU/ml)</td>
<td>0.544**</td>
<td>0.276**</td>
<td>0.296**</td>
</tr>
<tr>
<td>HOMA IR</td>
<td>0.214**</td>
<td>0.209*</td>
<td>0.392**</td>
</tr>
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*P < 0.05; **P < 0.001.
soluble receptor between lean and overweight subjects. In contrast to our results, the aforementioned study was performed exclusively in men, suggesting that gender plays a pivotal role in determining the levels of these soluble receptors (18).

In this study, the percentage of body fat was the best predictor of the levels of both soluble receptors in the overall study population as well as in the obese patients considered apart. This is in agreement with previous studies showing that BMI was the best determinant of sTNFα receptor levels (12, 13, 16, 17). In contrast, this is the first study to demonstrate that the levels of these receptors are higher in diabetic than in non-diabetic females, independent of the degree of adiposity. In fact, the presence/absence of diabetes was a significant predictor of both soluble forms of TNFα receptors.

Some authors have demonstrated a relationship between fasting insulin or HOMA IR and sTNFR1 (12) or sTNFR2 (12, 13) in obese patients. Fernández-Real et al. (17) further demonstrated a negative correlation between insulin sensitivity and sTNFR2. In our study, a positive and significant relationship was observed between both soluble receptors and insulin plasma levels or HOMA IR in the overall study population and this association was maintained for the sTNFR2 alone when the patient groups were assessed separately.

The increase in the levels of these receptors, and their relationship to the degree of insulin resistance, could be viewed as resulting from an increase in the production and activity of the TNFα system, secondary to an excess of adiposity, in order to breakdown the expansion of fat depots. Indeed, it has been observed that TNFα can decrease adipose tissue lipoprotein lipase activity (24, 25) and can also induce insulin resistance through its ability to produce serine phosphorylation of insulin receptor substrate 1, thus decreasing the tyrosine kinase activity of the insulin receptor (26). Both mechanisms are responsible for a fall in adipocyte and/or muscle tissue fuel bioavailability and of an increase in lipolysis (27).

Another interesting aspect of our study is the observation that there is a significant relationship between sTNFR1 or sTNFR2 and plasma leptin levels either when the overall study population is considered or only in the obese patient group. Other authors have shown bivariate correlations between leptin and sTNFR2 (18, 28) or both TNFα receptors (12) in obese patients or healthy and diabetic subjects. Further, when we included the values of leptin concentrations as an independent variable in the multiple regression analysis model, both leptin levels and the presence of diabetes were the only independent predictors of sTNFR1 and sTNFR2.

These observed relationships between the proteins suggest a certain degree of overlapping between leptin and TNFα. The rationale is that, first, both proteins are produced by adipose tissue in proportion to the amount of fat stores (10, 29) and are capable of regulating energy intake and expenditure (30). Secondly, a decrease in adipocyte expression and plasma concentrations of both proteins have been observed after weight loss (29) and these levels can increase during re-feeding/re-nutrition (10) and which indicates that similar mechanisms are involved in the regulation of these proteins. Finally, leptin synthesis can be modulated by the administration of TNFα in vitro (31, 32) and in vivo (33), although contradictory data have been published in the literature. An increase in the adipocyte production of leptin after TNFα incubation was observed by Kirchgeessner et al. (31). However, Zumbach et al. (33) found only a transient increase in serum leptin production with administration of TNFα and, recently, an inhibition in leptin synthesis was reported after long-term TNFα adipocyte incubation (32, 34).

Even though this study shows the largest sample of obese females to date and includes a group of severely obese patients, with or without diabetes, the study does have some limitations. First, the cross-sectional design of the study has not allowed us to infer causal relationships. In addition, the bioelectrical impedance analysis (BIA) prediction of body fat mass presents some methodological flaws especially in morbid obese patients. However, when the BMI is used instead of percentage of body fat, the same magnitude of relationship has been observed. Finally, due to the difficulties in recruiting lean type 2 diabetic patients, we are not able to state whether these relationships would still be maintained in this subpopulation.

To conclude, TNFα receptors are increased in obese and diabetic patients as a reflection of the degree of adiposity and associated insulin resistance. The presence of diabetes, adiposity or leptin levels are independent determinants of both sTNFα receptors. The independent association between plasma TNFα receptors and leptin levels in obese patients is consistent with the hypothesis that these proteins could be involved in the same pathway directed to regulate body adiposity. Further studies are necessary to elucidate the implications of the TNF–leptin system in the etiology and metabolic derangements associated with obesity.

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