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Establishment of a Cut-Point Value of Serum TNF-α Levels in the Metabolic Syndrome

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Cardiovascular diseases and type 2 diabetes are the major causes of mortality in Mexico. Metabolic syndrome (MS) is a cluster of factors that increase the risk to develop such diseases. Previous studies have shown that MS is associated with high tumor necrosis factor (TNF-α) levels. In fact, TNF-α has been proposed to be a useful marker for clinical diagnosis of inflammation at an early stage. Therefore, we analyzed TNF-α concentrations in Mexican individuals with or without MS and related these levels to the associated MS components. Clinical, anthropometric, and biochemical data were analyzed in 41 healthy and 39 MS individuals. Individuals were similarly grouped by age and gender. The serum TNF-α levels measured by a highly sensitive enzyme-linked immunosorbent assay (ELISA) kit were increased significantly in MS subjects compared with healthy individuals ($P < 0.001$). The assay showed 78.1% sensitivity and 61.5% specificity with a cut-point level of 1.36 pg/mL. TNF-α levels higher than the cut-point value were correlated with insulin resistance indices. These findings support the hypothesis that serum TNF-α concentration could be a useful marker for early MS diagnosis. Nevertheless, we suggest the establishment of specific cut-point values in each studied population to evaluate potential clinical applications. J. Clin. Lab. Anal. 23 : 51–56, 2009.

Key words: marker; diagnosis; insulin resistance; cytokine; TNF-α

INTRODUCTION

A high frequency of metabolic syndrome (MS) has been reported in Mexican populations (1,2). Because MS is predictive of cardiovascular disease and type 2 diabetes (3), conditions that are associated with high mortality rates in our country (4), early diagnosis in individuals displaying increased visceral fat, hypertension, hyperglycemia, hypertriglyceridemia, low high-density lipoprotein cholesterol (HDL-c) plasma levels, and insulin resistance (IR) is important.

Subcutaneous fat enhances cytokine production, especially that of tumor necrosis factor α (TNF-α), which interferes with insulin signal transduction (5,6). Increased levels of TNF-α have been reported in obese, MS, healthy hyperlipidemic, and diabetic subjects. The relative change in TNF-α levels depends on the clinical, genetic, and environmental backgrounds of a specific population, and the range of reference values remains controversial. Therefore, we measured the serum TNF-α

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concentrations in subjects with or without MS, in accordance with the National Cholesterol Expert Panel (NCEP) (7) criteria. We analyzed the interrelations between TNF-α concentrations and anthropometric and biochemical parameters as well as dyslipidemic variables and factors related to IR.

SUBJECTS AND METHODS

Subjects

This study was conducted at the Early Detection of Metabolic Syndrome Unit of the Health Sciences Campus of the Universidad de Guadalajara and involved 126 volunteers without previous diagnosis of MS or any other metabolic disease. The NCEP-III criteria were used to diagnose MS in these individuals (7). Thereafter, 80 subjects were selected and divided into two groups: (1) MS subjects with compatible clinical MS criteria ($n = 39$), and (2) control group subjects without clinical or laboratorial evidence of MS ($n = 41$). The male/female ratio was similar in both groups. All participants were age matched (40−78-years old). All individuals provided informed consent to participate in this study. In addition, all participants answered questions regarding their age, habits, and clinical and genetic backgrounds. None of the subjects was taking anti-inflammatory drugs or medications that affected metabolism or sympathetic nervous system activity.

Anthropometric and Blood Pressure Measurements

Using standard techniques, a nutritionist performed all anthropometric measurements. Standing height was measured to the nearest 0.1 cm using a fixed stadiometer, and body weight was measured to the nearest 0.1 kg. The body mass index (BMI) was calculated by dividing the weight (kg) by the square of the height (m^2). The waist circumference (WC) was measured midway between the lowest rib margin and the iliac crest, at the end of a gentle expiration. The index of upper body obesity, the waist to hip ratio (W/H), was calculated as WC (cm) divided by hip circumference (cm). The fat mass and percentage body fat were measured by bioimpedance analysis with a bioimpedance balance (Tanita Corporation of America, Japan). The systolic blood pressure and diastolic blood pressure were measured with an aneroid sphygmomanometer (Welch Allyn Tycos, Skaneateles Falls, NY), after a 10-min rest in the sitting position.

Obesity was defined as BMI $\geq 30\,kg/m^2$, overweight as BMI $\geq 25\,kg/m^2$, and underweight as BMI $< 20\,kg/m^2$.

Metabolic Evaluation

Blood samples were obtained from the subjects after a 12 hr overnight fast. Serum was separated by centrifugation of the freshly drawn blood and was immediately processed for measurement of the following biochemical parameters: total cholesterol (TC), triglycerides (TG), HDL-c, urea, creatinine, alanine aminotransferase, aspartate aminotransferase, and glucose. All biochemical measurements were conducted with commercially available kits (Biosystems, Spain), according to the manufacturers' recommendations. The concentration of low-density lipoprotein cholesterol (LDL-c) was calculated using the Friedewald Equation (8).

The homeostasis model assessment (HOMA) index was calculated as the product of fasting serum insulin and serum glucose divided by 405 (9). Another important metabolic marker, the TG/HDL-c ratio, was also considered. IR was defined as a TG/HDL-c concentration ratio > 3.0 (10). Hypercholesterolemia was defined as a TC level $> 200\,mg/dL$ and hypertriglyceridemia as a triglyceride level $> 150\,mg/dL$.

Insulin Determination

Blood samples were collected in a dry tube, and serum was separated. Serum was stored at $-20°C$ until the assay was performed. Determinations were carried out in duplicate, utilizing 50 μL of serum each.

Insulin determination was performed by immunoradiometric assay (IRMA kit, Immunotech, Czech Republic). Immunoreactive insulin (free insulin+insulin bound to anti-insulin antibodies) was measured. The antibodies in this kit exhibited no cross reaction with human proinsulin or C-peptide. Insulin concentrations in fasting healthy adults (2.1−22 μUI/mL) were considered normal.

TNF-α Measurement

Serum TNF-α was measured by a highly sensitive enzyme-linked immunosorbent assay in a commercially available kit (Biotrak ELISA system, GE Healthcare, UK). The assay was performed exactly as recommended by the manufacturer. The standards in this enzyme-linked immunosorbent assay (ELISA) procedure were calibrated to the WHO reference lot 87/650: 1 pg of Biotrak standard = 3 pg of WHO standard.

Statistical Analyses

The results were expressed as mean ± SD. Mean differences between control and MS groups were compared by Student's *t*-test. Anthropometric variables were analyzed independently by gender, and the independent *t*-test was used to compare the control

and MS groups. A χ^2 test was used for categorical parameters. A two-tailed P value <0.05 was considered statistically significant. The Statistical Package for the Social Sciences (SPSS for Windows, version 10.0.6, November 27, 1999) software was used for all analyses. To evaluate TNF-α concentration differences between control and MS subjects, the cut-point value, sensitivity, and specificity of the assay were determined by applying the receiver operating characteristic (ROC) curve.

RESULTS

After clinical, biochemical, and anthropometric evaluation of 126 individuals, we identified 39 subjects (31%) who fulfilled the NCEP-ATPIII criteria for MS. Consequently, we chose the control group ($n = 41$) from the 87 remaining volunteers without MS. Age and gender were matched between the groups. As a result, there was no statistical difference in mean age (49 vs. 51 years) or in the male/female ratio (0.41 vs. 0.39) between the control and MS groups, respectively.

The anthropometric parameters of both groups are shown in Table 1. Data are presented according to gender. All parameters were statistically different between the groups, with the exception of height in men and waist/hip ratio, body fat percentage, and fat mass in both genders (Table 1).

Table 2 shows a comparison of biochemical and clinical parameters between MS and control individuals. The mean values of systolic and diastolic arterial pressures, serum insulin, urea, HDL-c, VLDL-c, and TG were statistically different in the control and MS groups. In the MS group, all of these values were higher than the established cut-points, except for urea and insulin, which were within the normal ranges.

The serum TNF-α concentration was measured in both groups. As shown in Figure 1, a statistically significant ($P<0.001$) increase in TNF-α levels was observed in the MS group compared with the control group. The inter- and intra-CV percentage of the TNF-α assay was 86.6 and 20.7, respectively. An ROC curve revealed 61.5% sensitivity and 78.1% specificity for the determination of TNF-α levels, with a cut-point value of 1.36 pg/mL (Fig. 2).

We evaluated parameters related to MS, including TNF-α levels (Table 3). With regard to IR and metabolic indices, the mean values of the HOMA index and triglyceride/HDL-c ratio and the IR percentage (IR%; TG/HDL-c ratio >3.0) were examined. The IR% correlated well with the presence of MS and with TNF-α values >1.36 pg/mL.

The genetic background (family history of obesity, type 1 or 2 diabetes, acute myocardial infarction, cerebral vascular events, hypertension) and personal habits (smoking and alcohol intake, regular physical activity) were also analyzed. However, these parameters were not related to the presence of MS and/or increased TNF-α concentration (data not shown). Similarly, there was no significant association of hypercholesterolemia or hypertriglyceridemia with higher TNF-α levels (data not shown). In contrast, we found significantly higher ($P<0.05$) incidences of TNF-α values ≥ 1.36 pg/mL and HDL-c levels <35 mg/dL in the MS group compared with the control group.

DISCUSSION

The prevalence of MS in the Mexican population ($>30\%$) is higher than that reported for developed countries such as the USA (1,11,12). This high prevalence, in addition to the high mortality rates of diabetes and cardiovascular disease in Mexico (4), underlines the importance of finding markers and establishing cut-point values for known markers such as TNF-α for the early diagnosis of these diseases.

Several studies have described subclinical inflammation by the association of MS, obesity, and diabetes with high circulating TNF-α levels (13,14). In this study, the

TABLE 1. Anthropometric Indices of Control and Metabolic Syndrome Subjects

	Female		Male	
	Control ($n = 29$)	MS ($n = 28$)	Control ($n = 12$)	MS ($n = 11$)
Weight (kg)	71±11	79±11**	77±13	90±9*
Height (m)	1.59±0.05	1.56±0.06*	1.67±0.09	1.70±0.06
BMI (kg/m^2)	27.9±4.0	32.7±4.6**	27.5±4.2	31.0±3.2*
Waist circumference (cm)	89±12	101±18***	96±11	105±8*
Hip circumference (cm)	105±7	114±13***	102±5	107±6*
Waist/hip ratio	0.84±0.08	0.88±0.10	0.94±0.08	0.97±0.04
Body fat percentage	38.3±6.7	41.8±6.4	27.6±8.1	30.8±5.8
Fat mass (kg)	27.6±8.1	33.7±9.0	21.7±8.8	28.1±7.4

Mean value ± SD. BMI, body mass index. P value according to t-test: *$P<0.05$, **$P<0.01$, ***$P<0.001$ for comparison between control and MS groups in either gender.

TABLE 2. Clinical and Biochemical Values of Control and MS Groups

			CV (%)	
	Control ($n = 41$)	MS ($n = 39$)	Inter	Intra
SBP A/mmHg	118±9	134±11*	54.1	8.1
DBP A/mmHg	77±6	84±10*	39.1	10.0
Insulin (mUI/mL)	8±5	15±9*	272.9	65.7
AST (UI/L)	16±6	17±7	29.0	38.0
ALT (UI/L)	19±9	17±11	30.6	56.6
BUN mg/dL (mmol/L)	15±4 (5.35±1.42)	13±3 (4.64±1.07)*	90.4	24.7
Creatinine mg/dL (μmol/L)	0.90±0.36 (79.56±31.82)	0.92±0.26 (81.33±22.98)	11.4	35.1
Glucose mg/dL (mmol/L)	96±10 (5.33±0.55)	99±15.4 (5.50±0.83)	14.0	13.1
Total cholesterol mg/dL (mmol/L)	202±46 (5.23±1.19)	185±41 (4.79±1.06)	39.7	22.5
HDL-c mg/dL (mmol/L)	54±24 (1.39±0.62)	39±11 (1.01±0.29)*	86.0	23.1
LDL-c mg/dL (mmol/L)	124±9 (3.21±0.23)	112±10 (2.90±0.26)	46.4	36.5
VLDL-c mg/dL (mmol/L)	24±11 (0.27±0.12)	34±16 (0.38±0.18)*	164.7	47.4
Triglycerides mg/dL (mmol/L)	118±55 (1.33±0.62)	170±79 (1.92±0.89)*	163.1	47.3

Mean value ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood ureic nitrogen; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; VLDL-c, very low-density lipoprotein-cholesterol. P value according to t-test: $^*P < 0.05$ for comparison between control and MS groups.

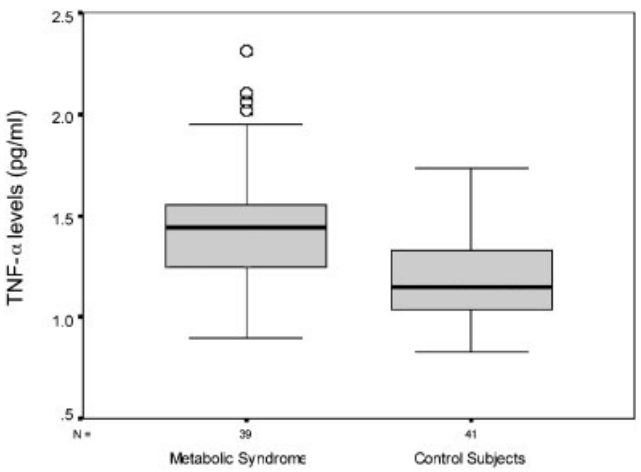

Fig. 1. Distribution of serum TNF-α levels in MS patients and control subjects.

serum concentration of TNF-α, an inflammatory cytokine implicated in IR, was analyzed in individuals diagnosed with or without MS. A significantly increased concentration of TNF-α was found in the MS group, similar to a previously reported study (15). Indeed, several reports indicate that certain dyslipidemias exhibit pro-inflammatory stages that are associated with elevated TNF-α levels (16–19). Nevertheless, in our study hypertriglyceridemic and hypercholesterolemic individuals did not exhibit increased TNF-α serum levels. On the contrary, low HDL-c levels were present only in MS patients with TNF-α concentrations higher than 1.36 pg/mL. Although we expected hypertriglyceridemia and low HDL-c levels to be associated with high TNF-α levels, not all MS patients with elevated TNF-α levels displayed such blood lipidic abnormalities. Although our study cohort was not large, the findings reported herein do not completely support the diabetogenic effects of TNF-α through an indirect stimulation of adipocyte lipolysis.

Overweight and obese individuals displayed TNF-α values greater than 1.36 pg/mL. On the other hand, all MS subjects with normal BMIs had lower TNF-α serum concentrations than the established cut-point value, supporting the essential role of obesity in TNF-α production (20). A nonindependent predictor of MS was WC; however, in this study a high WC was also found in control subjects according to NCEP-III and IDF criteria (7,21). Such findings are in agreement with reported results of Mexican populations, in which ~35.5–77% of nondiabetic subjects had increased WC values (1,2). Our study was limited in the analysis of other factors that affect TNF-α production, such as dietary fat intake (22).

Because age and gender affect several biochemical parameters as well as cytokine concentration, the study groups were matched by age and gender. Interestingly, we found that women with MS had significantly higher TNF-α levels compared with the control women, as described previously; however, TNF-α levels did not vary significantly between MS and control men (15). This finding suggests that TNF-α plays an important role in the development of IR in women.

We propose that similar studies should be performed to clarify the role of inflammatory cytokines such as TNF-α in MS. Furthermore, because high genetic heterogeneity has been reported in the Mexican population, TNF-α values should be analyzed in MS

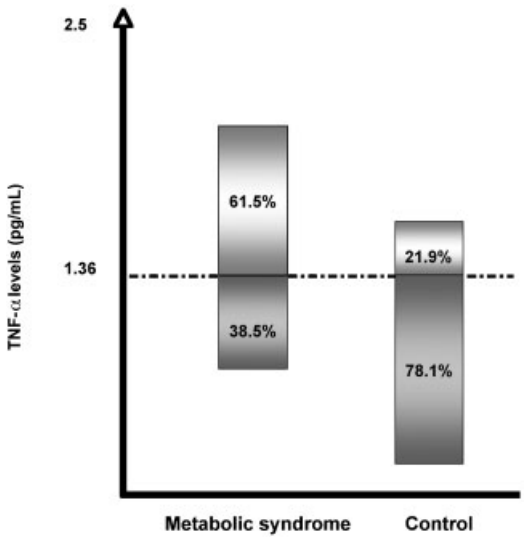

Fig. 2. Sensitivity and specificity of the determination of TNF-α levels in MS patients and control subjects, according to ROC curve.

TABLE 3. Markers of Insulin Resistance in Control and MS Groups

	Control ($n = 41$)	MS ($n = 39$)
TNF-α (pg/mL)	1.20 ± 0.21	$1.45 \pm 0.33^{**}$
HOMA index	1.9 ± 1.3	$3.6 \pm 2.3^{*}$
TAG/HDL-c index	2.58 ± 2.04	$4.80 \pm 2.62^{**}$
Insulin resistance (%)	31.7	74.4^{**}
TNF-α ≥ 1.36 pg/mL (%)	22.0	61.5^{**}

Mean value ± SD. TAG, triglycerides; HDL-c, high-density lipoprotein-cholesterol. Insulin resistance individuals with TAG/HDL-c indices >3.0. P value according to t-test: $^{*}P < 0.05$, $^{**}P < 0.001$ for comparison between control and MS groups.

individuals living in other regions of our country (22). Although a wide range of serum TNF-α levels has been reported in different populations, our results suggests a TNF-α cut-point value that is consistent with the recently reported range of control subjects. TNF-α levels of normotensive subjects were significantly lower than levels found in hypertensive individuals with MS diagnosis (23). Moreover, TNF-α levels differed between hypertensive subjects with or without MS. The measurement of serum TNF-α levels might be useful for the early identification of MS and of preclinical stages of chronic degenerative diseases. Therefore, our data clearly justify further analysis of the clinical utility of TNF-α in MS with significantly larger cohorts. In addition, the concentration of TNF-α receptors should be measured because these receptors modulate TNF-α bioactivity in MS patients (24). In addition, an analysis of genetic TNF-α polymorphisms associated with higher serum TNF-α levels, and the relationship of putative polymorphisms with MS in the Mexican population, should be undertaken.

In conclusion, this study suggests the clinical applicability of TNF-α serum levels as a predictive marker in the diagnosis of diseases linked to inflammatory processes, such as those that occur in MS.

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